Autoantibody Profiling of Cerebrospinal Fluid From Affective Disorders and Schizophrenia Patients by Phage Display: Method Optimization and Evaluation of Selected Autoantigens As Candidate Biomarkers

Thesis

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Autoantibody Profiling of Cerebrospinal Fluid from Affective Disorders and Schizophrenia Patients By Phage Display: Method Optimization and Evaluation of Selected Autoantigens as Candidate Biomarkers

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Abstract

Immunoproteomics approach applied on affective disorder and schizophrenia patient CSF samples yielded a number of candidate autoantigens. An overall analysis of those candidates revealed that impairment of myelination might have a role in etiology of such psychiatric disorders.

Validations were based on phage ELISA that is applied on the most prominent autoantigen candidate obtained from phage display selection. In parallel, a conventional ELISA by recombinant peptide rather then purified phage, helped reproduce the results that imply a tendency of autoimmune reactivity in patient CSF samples compared to controls. Comparison of these results with the closest family members of the candidate protein mapping to the same N-terminal sequence revealed that, the sequence obtained by phage display is more antigenic than its homologues.

Genomic expression profile of selected candidate and three other functionally related schizophrenia susceptibility genes were studied on mRNA in brain and spinal cord of mice, from postnatal day one until postnatal day forty-two. We could observe patterns of expression that may suggest a possible interplay of candidate proteins in the relevant neuronal maintenance mechanism.

Our approach using cDNA phage display used in the identification of autoantigens in inflammatory and autoimmune diseases of the CNS is quite feasible despite several drawbacks like high false-positive number.

Finally, our results may raise interest on myelination related proteins that may have roles in schizoaffective symptoms. However, strictly more effort has to be implemented to confirm and get more insight on our findings, for example developing mouse models and studying a broader range of serum and CSF samples.

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List of Abbreviations

Ag:	antigen
Ab:	antibody
AD:	affective disorders
APCs:	antigen-presenting cells
BBB:	blood brain barrier
BG:	background
BPD:	bipolar disease
CAMs:	cell adhesion molecules
CNS:	central nervous system
CNTNAP:	contactin associated protein or cell recognition protein, CASPR
CsCl:	cesium chloride
CSF:	cerebrospinal fluid
DISC:	discoidin
DDR1:	discoidin domain receptor 1
DS:	disease specific
EPI:	epilepsy
FACS:	fluorescence activated cell sorting
GAP43:	growth associated protein 43
Gas7:	growth arrest specific protein 7
GO:	gene ontology
HKG:	housekeeping gene
HPLC:	high performance liquid chromatography
Hsp60:	heat shock protein 60
ICD:	international classification of disease
IgG:	immunoglobulin gamma
IL3RA:	interleukin 3 receptor alpha
IPTG:	isopropyl-a-D-thiogalactopyranoside
PNS:	peripheral nervous system
RA:	rheumatoid arthritis
PD:	phage display
NPH:	normal pressure hydrocephalus
MRI:	magnetic resonance imaging
MARS:	multiple affinity removal system
MAG:	myelin associated glycoprotein

MBP:	myelin basic protein
MDD:	major depressive disorder
MHC:	major histocompatibility cells
MLD:	metachromatic leukodystrophy
MS:	multiple sclerosis
NGF:	nerve growth factor
NrCAM:	neuron-glia-CAM-related cell adhesion molecule
OND:	other neurological diseases
PCR:	polymerase chain reaction
PEB:	phage extraction buffer
PFC:	prefrontal cortex
PLA:	plaque lift assay
PLP:	proteolipid protein
PMP:	peripheral myelin protein
PND:	post natal day
RE:	restriction enzyme
RPL:	ribosomal protein
RZPD:	German Science Centre for Genome Research
Syn:	synuclein
SZ:	schizophrenia
SZAD:	schizoaffective disorder
TCRA/D:	T-cell receptor alpha/delta
T1D:	type 1 diabetes
ZNF:	zinc finger proteins

Summary

Major Depression and Bipolar Disease (BPD) are chronic affective disorders. Severe depression and BPD may exhibit psychotic symptoms like schizophrenia (SZ), implying the overall diffusion of psychiatric conditions. Genetic susceptibility risks and course of these disorders, as well as pathological mechanisms, are under extensive investigation, but are still elusive.

Today affective disorders and schizophrenic symptoms are usually controlled by lifetime commitment to certain mood stabilizers. Consequently, patients feel even more dejected due to the multiple side effects of the long-lasting treatment course of psychotropic medications or other neuroleptic treatments. Up to date, there are no reliable diagnostic biomarkers available for an effective diagnosis. Such a biomarker would ideally be able to differentiate the type and severity of an affective disorder or schizophrenia case with respect to other mood disorders and, thereby, offer a personalized therapeutic approach.

The fundamental hypothesis behind this work is based on the growing evidence showing elevated autoimmune responses associated with schizophrenic or bipolar disorder symptoms. The dissertation presented here is aimed at identification of biomarkers in the cerebrospinal fluid (CSF) of affective disorder and SZ patients, using an immunoproteomics approach with the major focus on phage display (PD) technology. PD is a molecular interaction study by which recombinant proteins are expressed on the surface of bacteriophages. PD technology is unique owing to two major factors: Firstly, the encoded nucleotide sequence of expressed proteins is easily accessible by a simple sequencing of phage DNA. Secondly, phages are

able to replicate in the bacterial host system, thereby vastly amplifying the captured interaction.

The thesis has evolved in three phases. The first is an empirical phase in which PD technology was modified in order to use CSF for autoantigen screening, and to set threshold criteria for discriminating real interactions from false-positives. This includes control experiments for a normal brain cDNA PD library, optimizations to upgrade the sensitivity of detection to minute IgG levels of CSF, and finally pilot selections on other diseases to spell out the background clones while getting familiar with the limitations of the technique.

The second phase mainly covered the selection of brain autoantigens by employing the modified biopanning protocol and characterization of these clones. Results referring to one particular candidate clone were highly motivating. Thus, a limited functional study on mice at different postnatal ages was included within the framework of the study.

The third phase mostly overlapped with the biopanning process and addressed the possibility of detecting candidate autoantigens directly from patient CSF without amplification of the signal through panning steps. In accordance, various ELISA protocols and a protein array were assessed and modified.

Chapter 1

Affective disorders, Schizophrenia and Autoimmunity

1.1 Introduction

Affective disorders are psychiatric disorders including bipolar, unipolar and schizoaffective disorders. Affective disorders have a remarkable social and economical impact reflected in the magnitude of research going on in order to understand the etiology and, thus, to improve diagnostic and medical approaches. Unfortunately, the causality and molecular mechanisms of bipolar disease and SZ remain obscure.

Bipolar and SZ affect the central nervous system (CNS) of individuals who experience first symptoms during early adulthood. These may involve delusions, irrational thinking and even hallucinations together with affective symptoms in a spectrum from mania to depression. Patients may exhibit complex and sometimes overlapping symptoms. Even though relapsing remitting cycles of symptoms are characteristic features of affective disorders, some patients suffer from a continuous disturbance.

Current diagnosis for affective disorders is assessed mainly by the Diagnostic and Statistical Manual of Mental Disease IV (DSM-IV) criteria via psychiatric counseling. Additionally, the patient's response to antidepressants is measured, although the mechanism as to how these drugs exert their action on affective disorders is still unknown.

1.2 Affective disorders

1.2.1 Unipolar (Major) depression (MDD)

Unipolar depression or major depression disorder (MDD) is clinically characterized by episodes of depression without a manic, mixed, or hypomanic phase according to the DSM-IV-TR criteria. The lifetime prevalence of unipolar MDD is around 10%. Women are twice more subject to MDD than men (Tsuang et al. 1994; Weissman et al 1996). Studies on twins and adoptions revealed that the heritability of MDD is 40-50% (McGuffin et al.; 1991, 2003; Sullivan et al., 2000; Torgersen 1986). A depressive patient may experience MDD with one single episode or it can be chronic with several relapses. MDD symptoms include a long-lasting feeling of sadness, insomnia or hypersomnia, loss or increase in appetite. Hallucinations, bizarre thoughts, desocialization, psychomotor retardation or agitation fall under severe symptoms. Having a wide spectrum of symptoms that resemble anxiety, schizoaffective, bipolar, and personality disorders or substance abuse render diagnosis of MDD rather complicated (Stein et al., 1995, Farabaugh, Mischoulon, Fava, Guyker, Alpert, 2004). Genetic epidemiology based on susceptibility genes is given below (Table 1.1).

Table1.1. Susceptibility genes for MDD

Candidate susceptibility gene for MDD	Name	Reference
Apolipoprotein E	APOE	Fan et al., 2006, Butters et al., 2003, Forsell et al., 1997
G-protein beta 3	GNB3	Bondy et al.,2002, Kunugi et al., 2002, Lee et al., 2004
Solute carrier protein family 6 member 3 (DAT)	SLC6A3	Frisch et al., 1999
Solute carrier protein family 6 member 4 (SERT)	SLC6A4	Furlong et al., 1998, Bellivier et al., 1998

1.2.2 Bipolar disease (BPD)

In the late 19th century, Emil Kraepelin defined BPD as a manic-depressive illness distinguishing it symptomatically from SZ. BPD is a mental condition with disturbances in mood shifting dramatically from depressive episodes to mania or hypomania, occasionally with a mixed state. A hallmark of BPD is the fact that symptoms usually merge during early adulthood with a devastating impact on the most productive period of life. Relapsing remitting cycles are characteristics of BPD with a great tendency to suicidal ideation during depressive episodes.

Bipolar disorders are categorized into 4 entities: bipolar I, bipolar II, cyclothymic disorder (bipolar I and bipolar II together in a rapid cycling nature), and bipolar disorder not otherwise specified, as described in DSM-IV (Bowden, 2002).

Co-morbidity of BPD with other disorders like attention-deficit/hyperactivity, substance abuse or mania is rather prominent. In addition, patients may present different symptoms according to subtype of the disorder, complicating and affecting the accuracy of diagnosis (Evans, 2000). Anxiety, for instance, is a highly frequent co-morbidity occurring in several studies (Henry C et al., 2003, Tamam L et al., 2002, Simon NM et al., 2003).

There are differences in presentation and the course of BPD between women and men, (Arnold, 2003, Christensen et al., 2003) although gender-dependent secondary factors tend to influence the response to medication and diagnosis more (Burt and Rasgon, 2004). However, a study by Hendrick et al., reports no gender difference in the 63 female and 68 male subjects studied, but nevertheless noted a higher risk of co-morbidity associated with women (Hendrick et al., 2000).

Kennedy et al. reported that male BPD subjects tend to have an earlier onset of mania and bipolar disorder than female (Kennedy et al., 2005).

Treatments with mood stabilizers like lithium, sodium valporate, or antipsychotic drugs (neuropileptics) like olanzapine are used to support therapy counseling. In the case of BPD, medication dependency is a lifelong commitment, which brings about multiple side effects over the years.

Epidemiology of BPD is unclear, yet a number of indications reveal a genetic predisposition. Twin, family, and adoption studies demonstrate the concordance ratio among monozygotic (MZ) twins to be 65%, compared to dizygotic twins with 14%. Thus the heritability of BPD is deduced to be up to 80% (Kendler et al., 1993). BPD and SZ both have 13q32 (Berrettini et al., 1994, Detera-Wadleigh et al., 1999) and 22q11 (Kelsoe et al., 2001) chromosomal regions as confirmed susceptibility loci. Together with evidences from genome-wide significant linkage studies (Lander, 1995), genetic epidemiology of BPD has overlapping findings that confirm a complex mode of transition involving may genes that predispose individuals to psychosis (Gottesman et al., 1967, McGue et al., 1989).

Table1.2. Recently characterized susceptibility genes for BP

Candidate susceptibility gene for BP	Name	Reference
G protein receptor kinase 3	GRK3	Barrett et al., 2003, Alexander et al., 2000
phosphatidylinositol-4-phosphate 5-kinase, type II, alpha	PIP5K2A	Stopkova et al., 2003
transient receptor potential-related channels	TRPC7	Nagamine et al., 1998, Yoon et al., 2001
putative cation-channel gene	MLC1	Verma et al., 2005

1.2.3 Schizoaffective disorders (SZAD)

Schizoaffective psychosis was coined by Jacob Kasanin in 1933. It is described as a more episodic psychotic illness compared to SZ or BPD, having both mood disorder and SZ-related symptoms of which affective symptoms are stronger than schizophrenic ones (Goodwin, 1990). ICD-10 Classification of Mental and Behavioral Disorders describes the schizoaffective disorder as an episodic disorder which presents both affective and schizophrenic symptoms prominently within the same episode of illness, usually simultaneously, but at least within a few days of each other. SZAD are given a separate category for diagnosis, because they are too common to be ignored. Subtypes of SZAD are, firstly, the bipolar type when manic and mixed states are involved, and secondly, the depressive type when depression is more pronounced. In general, a schizoaffective patient has a better prognosis than a schizophrenic, but worse than a bipolar patient. Usually anti-psychotic drugs and mood stabilizers are offered for treatment. Prevalence is not reported and there is no gender difference in the bipolar type of SZAD though women are more affected by the depressive subtype.

1.3 Schizophrenia (SZ)

SZ is a severe yet highly prevalent neuropsychiatric illness affecting 1% of the population worldwide. Patients suffer from a great decline in quality of life, along with high risk of suicidal attempts.

The symptoms usually initiate in early adulthood, between the ages of 25 to 30, mainly composed of delusions, hallucinations, depression and neurocognitive dysfunction. Today, the treatments are more experimental than rational with low efficiency and a great deal of side effects.

There are controversial reports concerning role of gender in SZ. Women have a later age of onset that may partially be due to the female sex hormone oestradiol acting as a protective factor (Riecher-Rössler A, 2000). On the other hand, it was claimed in a review by Goldstein that women with SZ experience affective disturbances more than men, and that the overall etiology is bound up with neuroendocrine and sex effects (Goldstein, 2006).

Genetics and developmental predisposition are risk factors supported by a lot of evidence (Mueser and McGurk, 2004). Genetic epidemiology studies on SZ revealed several susceptibility genes (Table 1.3) and chromosomal abnormalities. Many studies indicate a higher risk of developing SZ in genetically related people. Environmental factors influence the overall picture. This is demonstrated in twin studies with monozygotic twins revealing a concordance of only 50% and heritability estimates around 85% (Tsuang, 2000).

Table1.3. Recently characterized susceptibility genes for SZ

Candidate susceptibility gene for SZ	Name	Reference
Dystrobrevin binding protein 1	DTNBP1	Straub et al., 2002
Neuregulin 1	NRG1	Stefansson et al., 2002, Corvin et al., 2004
D-Amino-acid oxidase and D-amino-acid oxidase activator	DAO, DAOA	Chumakov et al., 2002
Discoidin domain receptor	DDR1	Roig B et al., 2007
Regulator of G-protein signaling 4	RGS4	Chowdari, 2002
Disrupted-in-schizophrenia	DISC	Blackwood et al., 2001, Millar et al., 2000, Miyoshi et al., 2003

Recent findings convey several changes in the neurotransmitter system in the CNS of subjects with SZ, including the GABAergic (Dean et al., 2000, Ohnuma et al., 1999), serotonergic (Dean et al., 1999), glutamatergic, dopaminergic (Tallerico et al., 2001), and cholinergic (Tandon, 1999, Taylor et al.,1998) systems revealed by postmortem studies.

Growing evidence suggests abnormalities in white matter integrity in SZ. The most recent report on a study conducted in adolescents, involving patients with a history of visual hallucinations, provided strong evidence of lower white matter integrity using high resolution imaging techniques (Ashtari et al., 2007). SZ has a wide range of proposed etiologies; therefore, some examples based on the selected literature are given in appendix A1.

1.4 Immune and autoimmune activity within the CNS

1.4.1 CNS immunity

The CNS is an immunologically specialized site as there is no lymphatic drainage. Moreover it is protected from the entry of large macromolecules like circulating proteins and cells by the blood brain barrier (BBB). For instance, circulating leukocytes cannot enter the CNS through the tight junctions of the BBB. In addition, cerebral capillary endothelial cells are not reactive to endocytosis and the expression of adhesive molecules (Ransohoff, Kivisäkk and Kidd, 2003). When a systematic immune response is induced, endothelial cells of the CNS upregulate the expression of cell adhesion molecules allowing activated T cells to cross the BBB (Figure 1.1). When an antigen is present and associated with MHC molecules, activated T-cells stay in the CNS. After antigen is eliminated, activated cells either leave the CNS or they die in-situ (Griffin, 2003).

Glial cells have important roles in maintaining CNS during and after an inflammation. There are three major glial cell types: Firstly, the oligodendrocytes that are responsible for the generation and maintenance of the myelin sheet that surrounds the axon of neurons. In case of an inflammation, they are indispensable for the repair mechanism. Secondly, the astrocytes that support neurons by producing neurotrophic factors, maintain the BBB and dispose of toxic substances. Finally, the microglial cells take over the job of macrophages. Microglial cells are bone marrow derived and become active when an injury occurs or, in the case of inflammation, by expressing MHC class I and class II molecules.

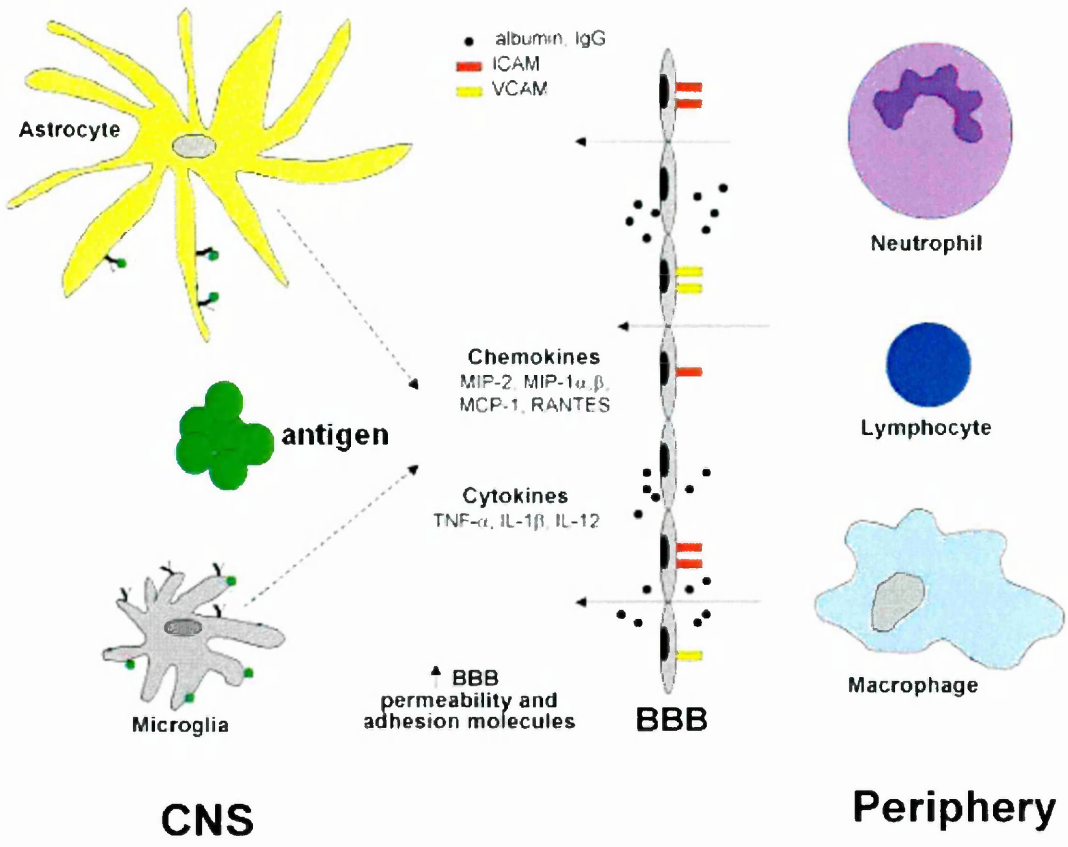


Figure 1.1 Mechanism that drives immune cells into CNS. Schematic overview of the initiation of CNS infiltration: A tight BBB (Blood brain barrier) is the key mechanism for immunologically privileged environment, simply blocking the entry of immunoglobulins and resting immune cells. In presence of an antigen that has a potential to activate astrocytes and/or microglia, these cells secrete pro-inflammatory cytokines and chemokines that prime immune cells in the periphery and endothelial cells of the BBB. Increased chemokine gradient attracts immune cells, while cytokines modulate expression of cell adhesion molecules on lining endothelial cells and initiate a migratory phenotype on attracted immune cells. Following the initial infiltration, contact of CD4+ T-cells with antigen presenting cells (APC) that result in formation of the right MHC-Antigen-TCR complex starts a cascade resulting in activation and proliferation of antigen specific T-cells in the CNS (modified from a review by Kielian T., Journal of Neuroinflammation 2004, 1:16).

1.4 2 Cerebrospinal fluid (CSF)

Cerebrospinal fluid (CSF) is produced from arterial blood of the lateral and fourth ventricles by a combined process of diffusion and active transfer. CSF circulates the brain and spinal cord, working as a buffer, nourishing and removing wastes and, at the same time, acting like a cushion protecting the CNS (Figure 1.2A).

In adults, the CSF volume is approximately 150 ml and it is produced at a rate of 0.2 - 0.7 ml per minute, 600-700 ml per day. CSF from the lumbar region contains 15 to 45 mg/dl proteins and 50-80 mg/dl glucose (2/3 of serum value). Protein concentration in cisternal and ventricular CSF is lower. Normal CSF contains less than 5 mononuclear cells per microliter and the white blood cell to red blood cell ratio is 1:700.

CSF is collected by lumbar puncture, during which a needle is cautiously inserted into the spinal canal between lumbar vertebrae L3/L4 or L4/L5 (Figure 1.2B). The maximum CSF that can be taken at a time is approximately 20 ml, whereby, for basic investigations, 4-8 ml CSF is sufficient. CSF used for analysis should look clear as a slightly red color indicates blood contamination that may cause considerable deviation of the IgG/albumin index and contaminate the CSF with serum proteins.

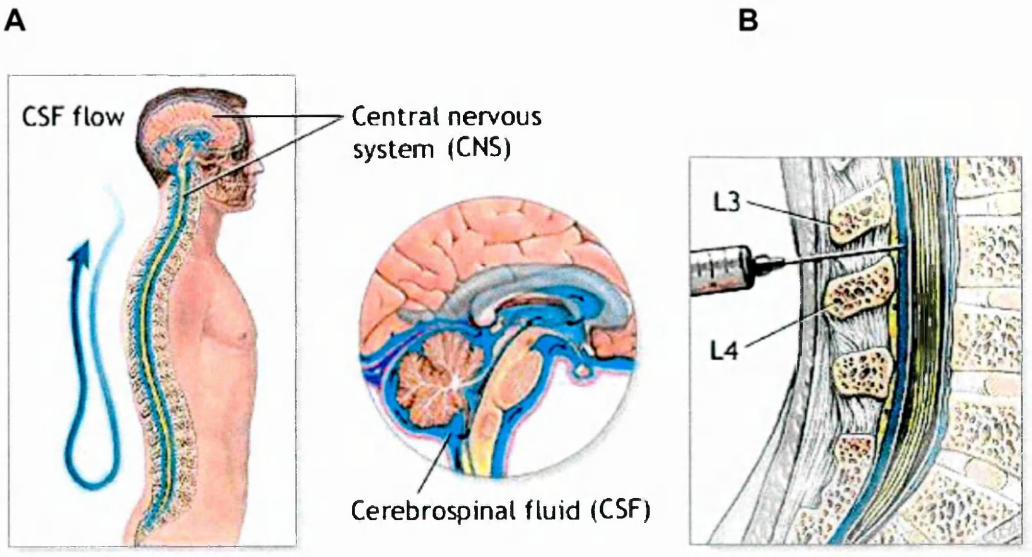


Figure 1.2 CSF and lumbar puncture. A. Cerebral spinal fluid (CSF) circulates from the ventricles through the subarachnoid space, protecting and nourishing the brain and spinal cord. **B**. CSF is collected by lumbar puncture (spinal tap). During the procedure, a needle is usually inserted between the vertebrae L3/L4 or L4/L5, the third and fourth lumbar vertebrae (figures modified from University of Wisconsin Hospitals and Clinics Authority and U.S. National Library of Medicine; (lumbar puncture as updated version by Bentley-Hibbert S., M.D., Ph.D., Department of Radiology, Weill Cornell Medical Center, New York, NY).

1.5 Immunological aspects in AFD and SZ:

1.5.1 From inflammation to autoimmunity

Inflammation is a consequence of the generalized innate immune response. Without inflammation, survival of the organism would be compromised. Due to this, it is a very effective yet non-specific defense mechanism, where many antigens are targeted. As explained earlier, CNS inflammation differs vastly from the periphery. However, CNS can get sensitized to inflammatory signals from the surrounding and initiate local responses. Microglia activation is an obvious sign of CNS inflammation that involves the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), chemokines (IL-8, MIP-1α, MIP-1β, MCP-1) and anti-inflammatory mediators, such as IL-1 receptor antagonist (IL-1ra) and TGF-β. Eventually, the pathogen is eliminated and activated immune cells, namely leukocytes, either leave the tissue or undergo apoptosis followed by the repair of the damaged tissues. Although this process is rigorously regulated, abnormalities related to inflammatory factors have roles in the etiology of various disorders including chronic inflammation, allergy and autoimmune disorders.

Autoimmunity is the aberrant immune response that occurs when immunological tolerance to "self" has failed. Learning to distinguish tissues and cells that belong to self from "non-self" (e.g. pathogens) occurs during maturation (ontogeny) of T-cells in the thymus. Immature T-cells that are not able to pass this vital assessment are eliminated. Nevertheless, there are always a number of immune cells that escape this filter. T-cells attacking self-antigens, so-called autoimmune T-cells, are constitutively suppressed by CD4+, CD25+, FoxP3+ regulatory T-cells if they escape thymic deletion. In other words, everybody has autoimmune T-cells that remain idle below the threshold of a pathological attack of autoimmunity. Indeed, such low-level autoimmunity is essential during development.

In addition, it is advantageous to the organism as it helps the immune system to function properly and to recognize neoplastic cells in cancer. Accordingly, autoreactive T-cells are particularly important in the CNS for neural maintenance and repair (Kipnis and Schwartz, 2005).

Autoimmunity is a concept far from being totally understood. One consensus is the fact that an autoimmune disease like lupus, multiple sclerosis, and rheumatoid arthritis affects women far more often than men. This phenomenon was explained by studies showing estrogens promoting, and androgens abrogating, the B-cell-mediated autoimmune diseases (Ahmed et al., 1999; Rider et al., 2001). However, this fact brings about a striking paradox as estrogen has a neuroprotective role demonstrated via in vivo and in vitro models (Behl et al., 2002, Sheldahl et al., 2007, Pozzi et al., 2006). Furthermore, a recent study indicates the neuroprotective effects of 17-beta estradiol E2 that promotes axon and myelin survival (Offner and Polanczyk, 2006). Furthermore, it has been demonstrated that autoimmune diseases have a geographical factor with a higher prevalence observed in the northern hemisphere and western community in contrast to a much lower frequency in countries with multiple infectious epidemics.

Autoimmune diseases are either systemic or localized. For instance, rheumatoid arthritis (RA) is systemic where joints are the targets; multiple sclerosis (MS) is local to the CNS; Hashimoto's thyroiditis affects the thyroid gland; in Type I diabetes (T1D, IDDM) insulin-producing beta cells in the pancreas are targeted. Autoimmune diseases are usually treated with immunosuppressive or anti-inflammatory agents, or replenishment of the eliminated metabolic components, for example, the insulin hormone in the case of T1D.

1.5.2 Evidence that supports inflammation and autoimmune hypothesis of AFD and SZ

A great number of studies have elucidated the role of inflammation and autoimmunity in several CNS-related diseases and psychiatric disorders, reporting various autoantibodies against CNS proteins in the sera, CSF and brain tissues from BP and SZ patients (Henneberg et al., 1993, 1994; Müller et al., 1992; Legros et al., 1985).

Regarding affective disorders, an increased IgG titer during the depressed state have been described in many studies similar to the findings supporting the inflammatory hypothesis for SZ with increased concentrations of immunoglobulin and proinflammatory cytokines in serum and CSF. For instance, interleukin-6 (IL-6), IL-6 receptor (IL-6R), IL-1R antagonist (IL-1RA) and IL-2R plasma levels, interleukin1 and interleukin 3 activity (Maes et al., 1995) are higher in SZ patients compared to controls. A function of IL6 is activating B-cells to produce antibodies, thus high levels of IL-6 may be part of an ongoing humoral response (Jones, Mowry, Pender and Greer, 2005). In this regard, elevated IL-6, interleukin-6 receptor (IL-6R), IL18, IL12 have been shown in schizoaffective patients with a bad prognosis, resistance to treatment and intensified symptoms with a longer duration of illness (Müller et al., 2000; Nunes, 2005; Lin et al., 1998; Altamura et al., 2003). In contrast, Clara cell 16KD protein (CC16), which is an endogenous protein with an anti-inflammatory and immunosuppressive effect, has lower serum concentrations in SZ patients (Altamura et al., 1999).

McAllister et al. have provided evidence of elevated levels of CD5+ B cells in approximately 30% of SZ patients. This was opposed by Ganguli et al., who confirmed other immune alterations but not an elevation of the CD5+ B

lymphocyte population for the characterization of a subset of patients with SZ (McAllister et al., 1989; Ganguli et al., 1993).

Epitope similarities to viral agents increase the risk of developing the disease when accompanied by an immune dysfunction. (Yolken and Torrey, 1995; Torrey et al., 1997; Tatsumi et al., 2002).

Evidence supporting the inflammatory hypothesis postulates that etiopathogenesis of some cases of SZ is based on altered levels of antibodies to cytomegalovirus, Toxoplasma gondii, and human herpesvirus type 6, provided that the patients with recent onset of SZ were untreated (Leweke et al., 2004; Yolken et al., 2001; Pelonero et al., 1990; Dickerson et al., 2003). It is shown that anti-psychotic treatment changes characteristics of inflammatory responses (Maes et al., 2000).

Interestingly, SZ has been associated with a reduced occurrence of other autoimmune diseases and malignancies. SZ patients and their first-degree relatives usually possess a lower risk of having another autoimmune disease (Eaton et al., 2006).

Similarly, Yovel et al. reported high natural killer cell activity in the serum of SZ patients, which may explain the low incidence of lung cancer and other malignancies in those patients, despite their higher rate of smoking (Yovel at al., 2000).

Other evidence reveals an impaired blood-brain barrier, besides immune dysfunction, in affective disorder patients, increasing their susceptibility for an autoimmune reaction. In addition, two independent studies have reported age and

gender to be relevant factors for autoantibody association with affective disorders (Hornig et al., 1999, 1996; Schwarz et al., 1998).

Excessive IgG transmissions from blood into the CSF, increased synthesis of IgG within the CSF, together with elevated monocytes and T lymphocytes, have been observed in some of the affective disorder patients (Breunis et al., 2003; Maes et al., 1993; Hornig et al., 1999). This activity results in aberrant antibody surveillance in the CNS. Such an unusual exposure to increased immune response may potentially result in mal-recognition of certain endogenous proteins within the CNS that resemble exogenous antigens. Similarly, self-peptides or cellular components that are normally out of scope of immune surveillance may become targets of humoral and cellular response. For instance, nuclear and phospholipid antigens (De Vries et al., 1994; Legros et al., 1985), myelin basic protein and nerve growth factor (Klyushnik et al.) are well-studied cases.

Detection of autoantibodies against the heat shock protein Hsp60 have gathered considerable interest as heat shock proteins are known targets in autoimmune diseases (Wang et al., 2003; Rothermundt et al., 2001; Schwarz et al., 1998; Kilidireas et al., 1992). However, one study reported that sera from MDD and SZ patients, and all normal controls, reacted similarly with Hsp60, and claimed that heat shock proteins are antigens of many pathogens and, thus, cannot be regarded as autoimmune specific indicators. Alternatively, their study yielded an 80-85 kDa antigen that was highly reactive with the IgG of 30% of SZ patient samples (Mazeh et al., 1998).

Direct evidence in favor of an autoimmune basis for AFD/SZ etiology would have been the presence of lesions in affected regions of the brain, or transmissibility of T cells or antibody to a healthy individual to create similar symptoms. There are no lesions detected in AFD/SZ patients. However, the transfer of purified IgG from patients to rats subsequently caused structural changes in the brain that are similar to SZ, while an identical experiment on monkeys created EEG disturbances (reviewed by Jones, Mowry, Pender and Geer, 2005).

On the other hand, a very important support for autoimmune basis for SZ was put forward by a study involving systemic lupus erythematosus (SLE), a known autoimmune disease that is characterized by autoantibodies against DNA. Patients with SLE develop SZ-like symptoms. This study showed that anti-DNA antibodies cross-react with N-methyl–D-aspartate receptor (NMDAR), which is a neurotransmitter receptor (Diamond et al., 2001, 2008)

Table 1.4 shows immunity related changes in the CSF of AFD and SZ patients, together with known autoimmune disorders SLE and MS.

Disorders	CSF findings	References
AFD/SZ	Elevation of IgG titer	Kirsch and Wyatt, 1991
	Autoantibodies	Sokol et al., 2007
	Elevated levels of monocytes and T lymphocytes	Nikkilä et al., 2007
	Elevated levels of cytokine IL1-beta	Levine et al., 1999
	Impaired blood-brain barrier	Kirch et al., 1992; Bauer and Kornhuber, 1987
SLE	Anti-nuclear autoantibodies	Diamond et al., 2001, 2008
	Elevated levels of intrathecal matrix metalloproteinases (MMPs)	Trysberg et al., 2004
MS	Elevated B cell response	Link et al., 1989, Baig et al., 1991
	CSF antibodies to myelin basic protein (MBP), myelin-oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG)	Górny et al., 1983, Catz et al., 1986, Xiao et al., 1991, Baig et al., 1991
	CSF antibodies to heterogeneous nuclear ribonucleoproteins (hnRNPs)	Sueoka et al., 2004
	Elevated levels of proinflammatory cytokines	Matusevicius et al., 1996

1.6 Biomarker Research and Immunoproteomics: Phage Display Strategy

1.6.1 Introduction

At the onset of any pathological disturbance, our body generates early biological warnings through peptides and other macromolecules. Translation of these signals into measurable, disease-specific markers, so-called biomarkers, ensures a better diagnosis and a personal medication tailored for each individual.

The immuno-proteomic survey of brain autoantigens as biomarkers originated from the assumption that an active immune system relays an elevated sensitivity towards self-proteins, in this case the CNS proteome, reflecting AFD and SZ pathology.

PD is the technology implemented for this quest. PD utilizes bacteriophages, viruses that target bacteria as a host organism. As a biological tool, phages are commonly used vectors to modify the *E.coli* genome in recombinant DNA engineering. A PD vector expresses the encoded segment of recombinant DNA as a protein or peptide besides the endogenous capsid components creating a fusion protein. A library with sufficient diversity of recombinant clones qualifies as a very powerful high-throughput selection tool. Furthermore, PD offers a similar condition to the interactive medium where reactions take place in solution.

PD is widely used in the ligand search for orphan receptors or vice versa, in signaling pathways, development of enzyme inhibitors and tags for protein purification. PD is favored especially in the development and epitope mapping of novel antibodies.

1.6.2 Biomarkers

Any biomolecule whose metabolic state indicates a disease condition can be regarded as a biomarker. Biomarkers in the proteomics field are proteins or peptides that have an altered expression, post-translational modification or other major changes that can be evaluated in conjuncture with a dysfunction or a disease.

The essential role of a biomarker is to define the personal risk of developing a disease in an objective manner and making it possible to take early measures, or at least, help early diagnosis before the major outbreak. Moreover, response to a given medical treatment or a new drug can be evaluated using a biomarker, in order to address people who are most likely to benefit from the treatment in question (Nolan, 2006).

Biomarker research has become a very popular discovery area as molecular biology, genomics and proteomics gain tremendous acceleration. Screenings and validations are faster, significantly more sensitive and precise than before, but still not perfect.

Blood tests are examples for commonly applied biomarker tests. For example, immunoglobulins against a previous infectious disease or a current epidemic are routinely measured from blood. Further examples are cardiovascular disease biomarkers like serum amyloid A protein, interleukin-6, interleukin-8, fibrinogen, and troponins, of which cardiac troponin I is elevated before a cardiac injury. The major cerebrospinal fluid (CSF) biomarkers in 90% of multiple sclerosis (MS) patients are oligoclonal bands solely in CSF, but not in serum. Similarly, measuring amyloid-beta peptide by ELISA is an indication for amyloid plaque formation in Alzheimer's disease.

1.6.3 Phage Display

1.6.3.1 Principle

In 1985, George P. Smith and collaborators initially described the PD concept (Smith, 1985; Scott and Smith, 1990; Smith and Petrenko, 1997), and soon it became one of the most powerful protein engineering tools. PD is a combinatorial technology for selecting peptides or proteins with specific binding properties as it provides a physical linkage between genotype and phenotype (Smith and Petrenko, 1997). Indeed, this shortcut made PD a unique approach for those who wish to study novel affinity partners in protein-protein interaction studies. Cloning a gene of interest as a nucleotide sequence that encodes the protein to be displayed into a phage genome enables expression of that protein or peptide as a fusion to phage coat protein. Another advantage is that it is possible to amplify a constructed library simply by a passage through a compatible bacterial host (Smith, 1985; Kay and Hoess, 1996).

An expedient phage library should be composed of diversity of coding sequences, potentially represented in a sufficiently large number of recombinants in the range of several million clones (Sidhu et al., 2000). An immobilized ligand is used to select the high affinity partners out of such a library in which 10 to 100 copies (copy number) from each recombinant clone are present.

After each biopanning, the stringency, based on the number and duration of washing cycles, is increased to an extent determined by the user. High affinity clones are eluted and amplified in a bacterial host system (enrichment). Thereby, a fraction of the primary recombinant collection is now represented in thousands of copies for the next biopanning round.

There are basically two classes of enrichments: firstly, matrix-to-phage (background enrichment) and, secondly, phage-to-phage (marker enrichment). Enrichment is assessed by an assay called titer assay where serial dilutions of phage are plated over agar plates using a soft-top agar mixed with bacteria at mid-log growth phase. As the phage infect and burst host cells, single plaques become visible, each representing one recombinant clone. The number of plaques corrected to dilution factor per milliliter gives the titer. Furthermore, the titer provides information about the number of viable phages (plaque forming unit or pfu) that are able to infect and propagate, which is a determinant for amplifying the selected phage for the next round (Lowman and Denise, 2004).

Theoretically, multiple rounds of iterative selection yield the highest affinity-binding clones. After the final round of selection, the amino acid sequence of the clone of interest can be obtained by sequencing the inserted DNA within the phage genome (Hoogenboom, 1997; Hoogenboom et al., 1998; Sparks et al., 1996).

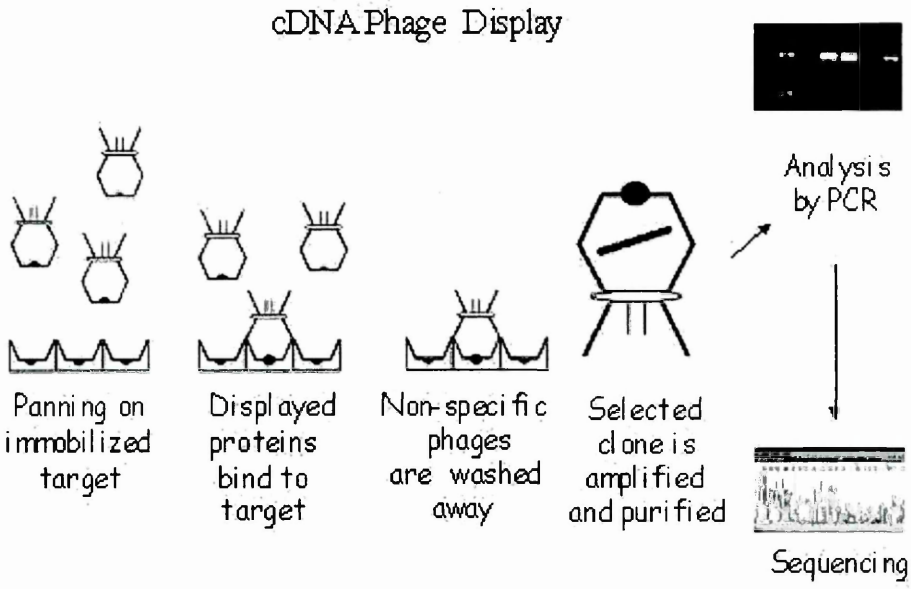


Figure 1.3 Panning cycles: After immobilization and incubating the PD library with the target, non-specific phages are washed away selecting strong binders bound to the target. Infecting the selected clones in *E.coli* for propagation follows using acidic or high alkali buffer for elution of the bound phage. Comparing input to output phage titers can monitor enrichment. Usually, three to four rounds of panning are sufficient to select clones with best affinity to the target and identity of the clones are obtained by DNA purification and sequencing. Illustration by Sayan-Ayata, E.

1.6.3.2 Applications of phage display technology

PD is a versatile technique that can be applied *in vivo, in vitro,* as well as *ex vivo* (Lee et al., 2002; Whaley et al., 2000; Johns et al., 2000; Sparks et al., 1996; McCafferty and Johnson, 1996; Odermatt, 2001). As a high throughput assay for protein-protein interactions, it has major contributions to many areas of biology and biochemistry, such as molecular and cellular biology, oncology, immunology, pharmacology and protein chemistry (Smith and Petrenko, 1997; Sidhu, 2000).

PD has been successfully applied in protein-protein interaction studies (Smith and Petrenko, 1997; Thom et al., 2006), epitope mapping (Wang et al., 2004; Enshell-Seijffers et al., 2003; Tarnovitski et al., 2006; Mumey et al., 2003) and drug and vaccine discovery (Wang et al., 2004; Ganglberger et al., 2000).

1.6.3.3 Types of phages used in phage display

Bacteriophages infect bacteria by inserting their genome for translation into viral proteins. Bacteriophages are classified as lysogenic (non-lytic) and lytic, according to the way they escape from the host. Non-lytic phage resides in the host with its DNA integrated but suppressed for expression of phage genome until a threat appears. It modifies host DNA polymerase and uses its DNA replication system to encode the viral genome. All phage proteins are produced before they assemble and burst out of the host cell.

M13 belongs to class of non-lytic bacteriophages, including fd and f1 phages that are most frequently used in PD systems. They have a circular single-stranded DNA genome within a tube of single major coat protein in several thousand copies of protein 8 (P8), and have four additional minor capsid proteins at the ends (P3-P6 and P7-P9) (Mullen et al., 2006; Sidhu, 2000).

Lytic phages, like lambda, T4 and T7, are ready to replicate their genomes and produce phage particles after they infect the host cell. When daughter phages are mature they erupt out of the host and spread to infect new bacterial cells. T even and T odd bacteriophages have linear genomes. The T7 phage head has an icosahedral shape and a small tail and infect gram-negative species of bacteria, like *E.coli* BL21. The T7 tail functions as an anchor between the phage and bacterial receptors for the transferring of the viral genome (Casjens and Sherwood, 1985).

Filamentous phage M13 was the first phage used in the development of PD (Smith, 1985). After successful implication of M13, several other *E.coli* targeting bacteriophages like lambda phage (Santini et al., 1998), T7 and T4 lytic phages

(Ren et al., 1998), and eukaryotic viruses like baculoviruses (Possee et al., 1997) were introduced. The non-lytic bacteriophages M13, fd, and f1, pIII, PVIII, PVI are often used in random peptide libraries and antibody display systems, whereas lytic phages are preferred in cDNA display systems.

1.6.3.4 cDNA display libraries

Complementary DNA (cDNA) is a DNA copy of the RNA template derived by the enzyme reverse transcriptase. A sufficiently large cDNA library reflects all the genes of the tissue that are being actively transcribed into mRNA at the moment of RNA isolation. Ideally, the degree of expression of a particular gene is directly related to the relative abundance of that particular mRNA (Smith and Petrenko, 1997).

Non-lytic phage systems like M13 require all components of the phage particles to be exported through the bacterial inner membrane before phage assembly takes place. This is not an issue for extracellular domains of receptors or secreted proteins, as they require disulfide bond formation. However, considering a total cDNA library where encoded products are folding in a reducing medium, the limitations become apparent as only a subset of total proteins can be displayed. Lytic phages, on the other hand, avoid this problem because the assembly takes place entirely in the cytoplasm (Jespers and Fransen, 2004; Danner and Belasco, 2001). T4 phage covalently displays the cDNA library on the C-terminus of its WAC tail and SOC coat proteins (Ren et al., 1996), whereas T7 uses the C-terminus of the major capsid protein 10A (Novagen T7 Select system).

1.6.3.5 T7 Select system

The T7 Select phage library (Novagen) from brain tissue is constructed in the T7Select 10-3 vector. Recombinant phage produces gene 10 proteins that have the carboxyl terminus fused to the peptide encoded by the cloned insert.

C- terminal fusion is an advantage for constructing a cDNA library as it prevents immature termination of translation.

The product of gene 10 is the major capsid protein of T7 phage particles and there are 415 copies of this protein per capsid. The phage particle assembles as a mixture of the normal capsid protein and the fusion protein.

Phage assembly takes place in the cytoplasm of *E.coli*. There are two strains of *E.coli* bacteria that are compatible with the T7 system: BLT5403 and BLT 5615.

In principle, capsid is composed of a large number of 10A proteins together with 5-15 copies of 10 B fusion proteins per virions in a 10-3 vector system. Peptides and proteins up to 1200 amino acids can be displayed in such a mid-copy phage system. When the fusion peptides are so large that the capsid cannot assemble within the host cell to make infective phage particles (because of the steric hindrance of the carboxyl-termini interfering with each other) 415 copies cannot fit in the space. Regarding BLT5403, 10A capsid protein expression is driven by the T7 promoter with the help of an ampicillin-resistant complementing plasmid providing extra copies of the 10A capsid protein. In contrast, BLT5615 expression is driven by the lacUV5 promoter and requires IPTG to induce capsid protein expression.

T7Select cDNA libraries are constructed in the mid-copy T7Select10-3b vector using a directional random primer cloning strategy. The cDNA library was constructed from mRNA extracted from normal brain tissue and then converted

into cDNA via random priming. Restriction digestion with EcoRI – HindIII, creates the reading frame of the inserted DNA with sticky ends. The vector expresses an inserted sequence in fusion to C-terminus of endogenous bacteriophage major capsid protein 10B at amino acid 348. Eventually, cDNA cloned into T7 phage genome is size-fractionated so that the smallest insert is 300 bp in length.

T7 is a lytic phage, meaning that the mature phages are released to the medium by rupturing the *E.coli* host. This is another advantage circumventing the complications of secretion through the cell membrane.

Furthermore, T7 is a rather robust bacteriophage, growing relatively fast and ensuring shorter durations for cloning and screening. Being still viable after treatments with 1% SDS, 5M NaCl, up to 4M urea, 10mM EDTA and acidic pH from 4 to 10, renders T7 very stable. The T7 system, therefore, offers great flexibility in adjusting washing and elution conditions (Table 1.5).

Table 1.5 T7 Select system.

OVERVIEW OF T7 SELECT SYSTEM		
T7 bacteriophage		Double stranded, linear DNA, lytic phage
Display options	High copy	415 copies per phage, up to 50 aa
		Short peptides up to 50 aa
	Mid copy	5-15 copies per phage, up to 50 aa
		Longer peptides up to 1200 aa
	Low copy	0,1-1 copies per phage, up to 50 aa
		Longer peptides up to 1200 aa (Maximum insert size: 3.6 kb)
Robust		5M NaCl, 4M Urea, DTT up to 100mM Alkali treatment up to pH: 10
Fast		Lysis in 2 h (lambda phage O/N)
		Plaque formation in 3 h
Pre-made normal brain cDNA library		1,67 x 10^7 pfu recombinant clones

1.7 Etiology of the control diseases used in the project

Several other disease control groups like epilepsy, normal pressure hydrocephalus and other (non-infectious) neurological diseases (OND) were used in the project. Using such diseases that are classified neither as autoimmune based, nor affective disorders as controls provided a broader view of the selected clone variability and a filter for false-positives. On the other hand, control samples from multiple sclerosis patients provided a list of selected clones from a well established autoimmune disease which demonstrated outcome of a selection from such pathophysiology.

1.7.1 Epilepsy

Epilepsy is a chronic neurological disorder characterized by recurrent seizures and affects around 0.5% of the population. The incidence is higher in underdeveloped countries (Hirtz et al., 2007). Epilepsy is defined by two or more unprovoked seizures and diagnosis is based on the medical history. The syndromes are likely to be associated with genetic and environmental factors, although the causality is still elusive, and the complete range of etiologies in the population is not known. Mutations in several genes that encode subunits of voltage-gated and ligand-gated ion channels have been associated with epilepsy (Meisler and Kearney, 2005). Anticonvulsants, surgery and ketogenic diet are methods of treatment.

1.7.2 Normal pressure hydrocephalus (NPH)

Hydrocephalus is a condition where CSF cannot be drained into the bloodstream when fresh CSF is produced and builds up exerting pressure on the brain. In case of Normal pressure hydrocephalus (NPH) draining of CSF is gradually blocked and excess fluid builds up over a period of time, generating pressure. NPH mostly becomes noticeable after 50 years of age. As a primary treatment, excess CSF

can at times be drained by means of a lumbar puncture. Symptoms may include reduced cognitive function, speaking, memory, and reasoning. Other common symptoms are lack of leg control and urinary incontinence. Similar symptomology to Alzheimer's disease, Parkinson's disease or dementia may lead to misdiagnosis. Due to this, symptoms can be misperceived, and misdiagnosed or go undetected for many years (Thynne, 2007).

1.7.3 Multiple Sclerosis

Multiple sclerosis (MS) is an immune-mediated disease of the CNS with relapsing–remitting symptoms causing neurological deficits in young adults. MS affects approximately 1 million people worldwide, mostly in developed countries, and has a higher prevalence in women compared to men. The etiology of MS is unknown. Appearance of symptoms and the disease course are variable among patients, although relapsing-remitting appearance of neurological symptoms is common. Due to destruction of myelin sheaths that insulate nerve fibers (demyelination), patients suffer from impaired cognitive and visual abilities, uncoordinated movements and disturbances in the sensory system. MS is diagnosed by magnetic resonance imaging (MRI) that shows lesions in the brain and spinal cord and the presence of oligoclonal Igs in the CSF.

1.7.4 Other neurological diseases (non-infectious) (OND): Somatoform disorder, cervical rhizopathy, and shoulder-arm pain

The somatoform disorders are a group of mental disturbances placed in a common category on the basis of their external symptoms. However, the physical symptoms present cannot be fully explained by a medical disorder, substance use, or another mental disorder. The DSM-IV-TR (4thEd, Text Revision) has a specific

category for somatic symptoms that includes somatization disorder, conversion disorder, pain disorder, hypochondriasis and body dysmorphic disorder. In order to meet the criteria for a somatoform disorder, the physical symptoms must be serious enough to interfere with the patient's daily life, and must occur unwillingly. Cervical rhizopathy and shoulder-arm pain are nerve root pains that may arise from injury of soft tissue. Neck and shoulder disorders are prevalent among both men and women. Work-related factors, both of physical and psychosocial origin, as well as lifestyle are contributing factors.

Chapter 2

Objective

Affective disorders (AD) reduce patients´ life quality dramatically and have a negative impact on the global health economy (Salokangas, 2007). BPD and SZ have high incidence ratios, yet the diagnostic accuracy is strikingly low (Bowden, 2001; Birnbaum et al., 2003). So far, underdiagnosis of BPD and SZ has repressed the effectiveness of the therapeutic approaches (Levine et al., 1997 and Evans, 2000). Despite all efforts, affective disorders and SZ remain ambiguous in diagnosis and cure, although they are the most common psychiatric disabilities. Therefore, like all other prevalent diseases, affective disorders and SZ are in urgent need of biomarkers.

Similar patterns of disease progression, repeating periods of regression and recovery, young age of onset, and comparable geographical incidence rates suggest a mutual molecular mechanism between autoimmune diseases and affective disorders. Further important features shared between AFD/SZ and established autoimmune disorders include; existence of oligoclonal bands in the CSF, elevated levels of IL2R, IL-6 and increased numbers of CD5+ B-cells in the serum (reviewed by Knight, Menkes, Highton and Adams, 2007).

CSF is the investigational material, preferred due to its proximity to the CNS while exhibiting a relatively less challenging composition compared to serum. In addition, autoimmunity related pathological changes are detectable in the CSF of AFD/SZ patients, as explained in the chapter of introduction, with several evidences from literature.

The CSF proteome reflects the dynamic physiological state of the central nervous system (CNS) making it very attractive for biomarker research on neurological and psychiatric disorders.

On the other hand, CSF differs from serum in the sense that the sample volumes are strictly limited, and procuring control CSF from healthy individuals is extremely challenging (Turck, 2005). Additionally, the immunoglobulin component of a standard CSF sample is approximately one thousandth of serum. For an autoantibody survey, such limitations may be intractable when taken together with the assumption that very few copies of a given disease-specific antibody are likely to be present within CSF or serum (Monaci and Cortese, 2004).

Immunoproteomics, mainly based on PD, is a convenient approach in the quest for brain autoantigens as it integrates high selectivity of the immune system to systematic monitoring of the proteome at a given physiological state (Turck et al., 2005). PD is capable of resolving CSF-related limitations as it benefits from the amplification step that enriches signals from phage displaying disease-specific autoantigen.

In addition, the PD strategy is rather straightforward in defining partners of molecular interactions owing to the short cut between the phenotype expressed on the phage and its genome (Smith and Petrenko, 1997). Finally, PD has proven useful in biomarker research; for instance, in validation of novel proteins for therapeutic intervention, in high throughput screening in the process of developing agonists or antagonists, and in mapping epitopes (Sidhu, 2001).

To further improve the overall sensitivity, the PD protocol applied in this work was especially modified for CSF samples with a very low immunoglobulin content that would render targeted autoantibody repertoire rather diluted.

Based on the given rationale, the ultimate goal of the thesis was to address the imperative necessity for disease-specific biomarkers for AFD and SZ.

In summary, aims of the thesis in chronological order were as follows:

1- Adaptation and optimization of phage display technology for autoantigen detection in the CSF of AFD and SZ patients,
2- Identification of biomarker candidates by screening AFD/SZ versus control CSF of EPI, OND and MS patients, using the optimized PD immuno-proteomics approach,
3- Evaluation, verification and further studies of the findings by alternative approaches like protein array, ELISA and brief functional studies.

A thorough investigation of the autoantibody repertoire of CSF by PD holds the potential, not only to unravel disease-specific candidate biomarkers, but also to contribute to a better understanding of autoimmune characteristics of AFD and SZ.

Chapter 3

Materials and Methods

3.1 MATERIALS

3.1.1 BUFFERS AND REAGENTS

a. MAIN BUFFERS

Phosphate buffered saline (1X PBS)

150mM NaCl, 9,1mM Na_2HPO_4, 1,7mM NaH_2PO_4,

Add distilled H_2O to 1 liter. Adjust pH to 7.4

Tris buffered saline (1X TBS)

150mM NaCl, 10mM Tris-HCl, pH: 7.5 Add distilled H_2O to 1 liter

b. ELISA BUFFERS

Elisa coating buffer

1x PBS or 0.05 M carbonate-bicarbonate

Elisa washing buffer

1X PBS + 0.05% TWEEN-20

Elisa blocking buffer

1X PBS-T, 1% BSA

Elisa substrate buffer

TMB (Tetra-methyl-benzidine) substrate solution (Sigma Aldrich)

Elisa stop solution

1M H_2SO_4 or commercial stop solution (Sigma Aldrich)

c. VIVASPIN and MARS

Vivaspin: MW5000 cutoff

Buffer A: 25mM KH_4PO_4, 500mM NaCl, 0.02% NaN_2, pH: 7,4

Buffer B: 2M Urea, 0.5M Glycine, pH:2.5

d. BIOMAG BEADS

Coupling buffer: PBBS 1%

1 X PBS, 1% BSA, 1mM Na_2EDTA

Washing buffer: PBBS-T 0,1%

1X PBS, 0,1% BSA , 0,1% v/v TWEEN-20

e. PANNING

Library coupling buffer: PBBS-T 0,1%

1X PBS, 0,1% BSA , 0,1% v/v TWEEN-20, sterile filtered (0.2mM)

Washing buffer:

1X PBS (or 1X TBS), 0,1-0,2% TWEEN-20 (0.2mM)

Elution buffer:

5M NaCl, sterile filtered (0.2mM)

PEB (phage extraction buffer) :

100mM NaCl, 20mM Tris-HCl, 6mM $MgSO_4$

f. PCR ELECTROPHORESIS BUFFERS

TAE Buffer (Tris-Acetate-EDTA) 50x

242 g Tris base

57.1ml Acetic acid

100ml 0.5M EDTA

Add ddH_2O to 1 liter and adjust pH to 8.5

g. MEDIA and SUPPLEMENTS

LB

10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, ddH2O up to 1 liter,

pH: 7.5, autoclaved

TB

12g Bacto tryptone, 24g yeast extract, 4ml glycerol, ddH2O up to 1liter, autoclaved

LB Top agar

1g Bacto tryptone, 0,5g yeast extract, 0,5g NaCl, 0,6g Agarose, ddH2O up to 100ml, autoclaved

20x M9 salts

20g NH_4Cl, 60g KH_2PO_4, 120g $Na_2HPO_4 .7H_2O$, autoclaved

M9LB

5ml 20X M9 salts, 2ml 20% glucose, 0,1ml 1M $MgSO_4$

h. Bacterial strains

BLT5615:

An *E.coli* host strain for the growth of the bacteriophage T7Select10-3. It needs an exogenous ampicillin-resistant plasmid for expressing the capsid protein. Production of 10A capsid protein is controlled by lacUV5 promoter, hence must be induced by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG).

BLT5403

An *E.coli* host strain for the growth of the bacteriophage T7Select10-3. It needs an exogenous ampicillin-resistant plasmid for expression of 10A capsid protein driven by T7 promoter. BLT5403 plaques are smaller and often accumulate deletions in the capsid fusion gene if the product is larger than 600 amino acids. Lysate titers are equivalent to BLT 5615.

3.1.2 ANTIBODIES

Table 3.1 List of antibodies used in optimization and control experiments.

Antibody	Type	Company	Dilution	Secondary-Ab conjugate	Control peptides
Anti-alpha-Synuclein	Monoclonal (clone211)	Sigma Aldrich	Used as test antibody at variable concentrations, In ELISA, 1:100	Anti- mouse HRPO 1:50000	-
Anti-β–Actin	Monoclonal	Alexis Biochemicals	1:1000	Anti- mouse HRPO 1:2000	-
Anti-Caspr	Polyclonal	Santa Cruz Biotechnology	-	-	N-terminal aa20-70
Anti-Caspr2	Polyclonal	Santa Cruz Biotechnology	-	-	N-terminal

3.1.3 PRIMER AND PROBES

CASPR4

Fw. Primer 5'- CACCATGCACCATCACCATCACCAT-3'

Rev. primer 5'-TTATCAATCTCTTCTATTCAGCCTTGCAAA-3'

CASPR1 mouse

Fw. primer 5'- GCTGGACACCATTCTACCAAA -3'

Rev. primer 5'- GGATGTAGCGAGCCGTAAAG -3'

Probe 5'- FAM-AATGCGACCTTCTTCGGGAATGTCA-TAMRA -3'

Amplicon size: 111

gDNA amplicon size with primers: 493

CASPR4 mouse

Fw. primer 5'- CTTCTGAGCTATCCAGCAGTCA -3'

Rev. primer 5'- CCACTGGTATTTGTTGGACACA -3'

Probe 5'- FAM-ATCGAAGAGACGGAGCTGGTGGC-TAMRA -3'

Amplicon size: 101

DDR1 mouse

Fw. primer 5'-CAGCTCCTGGTCGGACTCT-3'

Rev. primer 5'-TCTTCTTTGGGGAACACAGG-3'

Probe 5'-FAM-AGCAGGCTGGAAAGCAGTGATGGAGAT-TAMRA-3'

Amplicon size: 102

gDNA amplicon size with primers: 609

MBP mouse

Fw. primer 5'-GACCCAAGATGAAAACCCAGT-3'

Rev. primer 5'-GGCTGTCTCTTCCTCCCTTC-3'

Probe 5'-FAM-TCTTCAAGAACATTGTGACACCTCGAACAC-TAMRA-3'

Amplicon size: 95

GAPDH mouse

Housekeeping gene used as internal control (purchased from ABI Biosystems)

3.1.4 CSF SAMPLES

SZ, BP, MDD, NPH and Epilepsy CSF samples were obtained from the CSF Bank of Max-Planck Institute of Psychiatry, Munich, Germany, according to the ethical rules of the CSF bank. CSF samples for MS and other neurological diseases (diagnosed as somatoform disorder, cervical rhizopathy, parainfectious headache and shoulder-arm pain) were obtained from the University of Munich clinic. In optimization and pilot selections, additional 2 major depression samples and 2 normal pressure hydrocephalus control samples were used. After lumbar puncture was carried out by the medical staff, CSF samples were immediately stored at -80°C in aliquots at the clinic of the Max-Planck Institute of Psychiatry CSF bank. Tables 3.2-3.4 show the list of CSF samples and important patient data, while a more detailed version is given in appendix A2.

Table 3.2 List of CSF samples from bipolar disorder and schizophrenia patients

Sample	Gender	Age	Diagnosis	[IgG] µg/ml	Albumin Quotient*
369/98	F	43	Schizophrenia (SZ)	53	7,3
454/00	F	34	SZ	22.7	3,2
464/02	F	24	SZ	45.6	9,3
244/04	F	61	SZ	23.3	4,6
076/97	M	50	SZ	24.6	5,8
166/02	M	56	SZ	28.7	6,6
071/02	F	52	Bipolar disorder (BPD)	25.4	5,9
365/04	F	48	BPD	20.3	4,5
131/05	F	64	BPD	18.9	3,1
203/05	F	46	BPD	11.9	3,1
160/98	M	21	BPD	14.7	2,5

a. Demography of SZ and BPD patients:

Females:	(N= 8 – 72,7%)	Mean age: 46,5	SD: ±13
Males:	(N= 3 – 27,3%)	Mean age: 42,33	SD: ±19
All:	N=11	Mean age: 45,36	SD: ±14

Table 3.3 List of CSF samples from multiple sclerosis patients

Sample	Gender	Age	Diagnosis	[IgG] µg/ml	Albumin Quotient*
70004	F	41	Multiple Sclerosis (MS)	84.2	8,7
06705	F	40	MS	52.3	6,4
14905	F	43	MS	44.7	4,6
15005	F	40	MS	108	5,7
24605	F	26	MS	54.9	6,3
24705	F	31	MS	151	8,8
31205	F	45	MS	59.4	6,5
35305	F	39	MS	51.2	6,3

b. Demography of MS patients:

Females: (N= 8 – 100%) Mean age: 38 SD: ±6,4

Table 3.4 List of CSF samples from control disease patients

Sample	Gender	Age	Diagnosis	[IgG] µg/ml	Albumin Quotient*
014/00	F	25	Epilepsy (EPI)	26.6	4,3
300/04	F	26	EPI	15.5	2,9
339/04	F	56	EPI	61	7,2
477/01	M	17	EPI	34	4,8
326/02	M	70	EPI	62.3	8,9
330/04	M	46	EPI	31.9	5,5
053/05	M	23	EPI	21.1	3,6
01805	F	39	Headache	23	5,1
19405	F	45	Somatoform disorder	34.1	8,2
25505	F	41	Cervical rhizopathy	17.3	4,3
41405	F	42	Shoulder-arm pain	26.6	7,6

c. Demography of control disease patients:

Females:	(N= 7 – 63,6%)	Mean age: 39	SD: ±11
Males:	(N= 4 – 36,4%)	Mean age: 39	SD: ±17
All:	N=11	Mean age: 39	SD: ±25

* Albumin Quotient =Q(CSF/Serum)x10^3

3.1.5 Animals

For each postnatal day time-point (PND1-PND7-PND14-PND21-PND28-PND35 and PND42), 3 female and 3 male mice with C57BL/6 genetic background were used. Animals older than PND14 were obtained from Charles River WIGA GmbH (Sulzfeld, Germany). Others were inbred and maintained in the animal facilities of the Max Planck Institute of Psychiatry, Munich, Germany. Brain and spinal cord tissues were immediately frozen in liquid nitrogen after removal. They were stored at -80°C until cryosection or grinding for RNA extraction.

3.2 METHODS

3.2.1 cDNA Phage display assay

The T7Select normal brain cDNA library peptide library (Novagen) was panned against CSF immunoglobulin (IgG) using the modified PD protocol. CSF IgG is affinity captured onto the BioMag anti-human IgG-Fc beads. To begin each panning experiment, $1.7x10^6$ PFU of T7 normal brain cDNA phage with 100 copy number was used. For each subsequent panning, lysate obtained by amplification of the previous eluate was used. As a negative control, normal human serum (Sigma Aldrich) was coupled to BioMag beads and selected over negative lysate (empty phage).

1. Preparation of the beads

BioMag-anti human IgG-Fc specific beads (Qiagen) were used to capture CSF IgG repertoire. The volume of BioMag beads sufficient to capture all IgG was calculated for each patient depending on the IgG concentration given by the clinical information sheet. The beads were prepared by washing with phosphate buffered saline, 0.1% Tween20 (PBS-T) 3 times, each time carefully removing the supernatant after collection of the beads in a magnetic stand (Dynal). Eventually the beads were re-suspended in the original volume.

2. Negative selection of nonspecific clones

In order to clarify the library from the clones that have affinity to magnetic beads, T7 normal brain library was combined with BioMag anti-human IgG specific against Fc region, in a 1ml final volume of coupling buffer (PBBS-T, 0.1%BSA, 0.1% Tween 20) and incubated at 4°C for O/N. Following this, the library was recovered from the beads for biopanning.

3. Biopanning

Round 1

In parallel to clarifying T7 library, another set of BioMag beads (equal volume) were incubated with 5µg CSF IgG at 4°C, O/N. In all incubations with BioMag beads, a Dynal rotator or a rolling rotator (Dynal Biotech, Invitrogen) was used in mild rotation to avoid sticking and settling of the beads. Next, the beads, coupled to CSF IgG, were washed 3 times with PBS-T (0.1% Tween-20) and mixed with the clarified library for incubation at 4°C, O/N. The reaction was then rinsed 1 time 5ml of washing buffer PBS-T (0.1% Tween-20) and washed 2 more times for 10 min at room temperature (RT) in a 15ml falcon tube. After washing, bound phage was briefly rinsed with ddH_2O while being transferring to a clean 2ml Eppendorf tube, and eluted by suspending CSF IgG-phage-bead complex in 200µl of elution buffer (5M NaCl) at room temperature for 25 min. The elution mixture was placed in a magnetic stand and collected in a clean tube. Determination of the titer of the eluate and the lysate from bacteria was performed as described in section 4.

Round 2

A second round of panning was performed using amplified phage (in the range of 10^{10}-10^{11} PFU) recovered from the first-round eluate. Coupling of the BioMag beads to CSF IgG was performed the day before adding lysate from the first panning. Note that in the second round panning, lysate was used directly, without clarification. The same volume of beads were prepared, as described above, and incubated O/N at 4°C in the rotator.

To reduce nonspecific binding, the stringency was turned up by increasing the duration of each wash to 30 min with the same buffer configuration. The titer of the eluate and the lysate from bacteria was determined as described in section 4.

Round 3

The third round of panning was performed using amplified phage (in the range of 10^{10}-10^{11} PFU) recovered from the second-round eluate. Again, lysates were used directly without a prior incubation with empty beads alone. The same volume of beads was prepared, as described above, and incubated for O/N at 4°C in the rotator. To reduce nonspecific binding, the stringency was increased by using 0.2% Tween-20 for 30 min. The third round of panning was carried out using amplified second-round eluate (10^{10}-10^{11} PFU). Following titer assay for the third-round eluate, single plaques were amplified and prepared for sequencing as described in sections 6 and 7.

4. Titer assay

250µl aliquots of mid-log-phase *E.coli* BLT5403 cells with 0.5 optical densities at 600 nm were infected with 100µl from serial dilutions of T7phage. For eluates, serial dilutions were made ranging from 10^3 to 10^6 pfu/ml whereas, for lysates, dilutions were typically between 10^6 to 10^{10} pfu/ml. 7ml of top agar at 40 °C was

mixed with infected bacteria and poured onto 100-mm plates of LB agar supplemented with ampicillin to 20µg/ml (when using agar plates that are stored at 4°C, they should first be equilibrated to RT). Diligent and careful handling was essential to reduce the risk of cross-contamination. The plates were incubated at 37°C, and it took around 3 h (O/N at RT), until the plaques were large enough for counting and picking. Clear plaques demonstrated the lysis of the bacterial cells. Well-separated plaques were counted to determine the titer and picked for individual amplification for the subsequent analysis when necessary. Plates were stored at 4°C, inverted, with parafilm wrapped until plaque picking. The titer was calculated by (number of plaques x dilution factor) / (volume plated, in ml).

5. Infection of *E.coli* cells and enrichment

Typically, a T7 phage eluate had a pfu of 10^3 to 10^5 pfu on average. To amplify phage for the next round of panning, 100 µl of the eluted phage was used to infect mid-log BLT5615 *E.coli* cells in M9LB (minimal medium) supplemented with 1mM IPTG (isopropyl-a-D-thiogalactopyranoside) and ampicillin to 20 µg/ml. Additionally, in-situ amplification was performed for the first and second rounds of panning. This modification helped rescue high affinity binder clones with slow dissociation times. For this purpose, the beads were transferred into the infection tube after the eluate was collected from them. For this, 1ml aliquot from an uninfected bacterial culture was added to the bead pellet to resuspend and transfer them back into the culture. After infection, the culture was kept on the bench for 10 min to ensure adequate attachment of phage on the host cells. Next, the culture was placed in the shaking incubator (Innova 4230), at 37°C with vigorous shaking. In general, lysis took place after 2 to 3 h with clear signs of cell burst, such as culture becoming transparent and white strings of disrupted cell debris becoming visible.

The lysates were transferred into 2ml Eppendorf tubes and centrifuged at 10000 rpm for 10 min and the supernatant was stored as the lysate.

6. Analysis of clones:

a. DNA purification and PCR

To study single clones, randomly picked phage plaques were excised into 96 deep-well microtiter plates with 50µl phage extraction buffer (PEB), using sterile pipette tips. Only well-isolated plaques were picked in a perpendicular direction to the plate as phage plaques diffuse horizontally. The plaques were allowed to dissolve in the PEB buffer for an hour at RT, keeping the tips in the well, on an orbital shaker with mild rotation. Tips were removed after plaques were ejected into and mixed with the PEB buffer. 25µl from each phage extract was transferred into a microtiter plate for DNA purification. The rest was covered with a sealing mat and stored at 4°C (master clone plate).

In order to disrupt the phages, 25µl of dissolved phage extract were mixed 1:1 with 10 mM EDTA pH 8.0 in a microtiter plate and placed at 65°C for 10 min in a thermocycler. After cooling back to RT, the microtiter plate was centrifuged at top speed for 3 min and supernatants were collected in a clean microtiter plate, covered with a sealing mat and stored at 4°C (clone-PCR plate). Later, a conventional Polymerase Chain Reaction (PCR) was performed to amplify the DNA. A control reaction, without the template DNA, was always included to confirm the absence of contamination. PCR master mix was prepared and the reaction was programmed in an MJ Research P200 thermocycler as follows:

Reaction volume	50µl

10X Buffer, with 25mM $MgCl_2$	5µl
dNTPmix (10mM: each dNTP)	1µl
T7 Select up primer *	1µl
T7 Select down primer *	1µl
ddH_2O	39.75µl
Taq polymerase	0.25µl
Phage lysate	2µl

Primers (* stock: 5pmol/µl)

T7 up primer	(Novagen)	5'-GGA GCT GTC GTA TTC CAG TC-3'
T7 down primer	(Novagen)	5'-AAC CCC TCA AGA CCC GTT TA-3'

PCR Program:

80°C	5 min	Initial denaturation	
95 °C	30 sec	Denaturation	
59 °C	30 sec	Annealing	
72 °C	1 min	Elongation	33-35 cycles
72 °C	5 min	Final elongation	
4°C-12°C	Infinite	Cooling	

For clone analysis and restriction enzyme fingerprinting, 10µl PCR reactions were sufficient. Afterwards, selected clones were amplified in a larger volume (50µl PCR reaction). PCR products were stored at 4°C until purification.

b. Restriction fingerprinting directly after PCR

PCR products can be directly digested after an amplification reaction only if the PCR buffer does not interfere with the restriction enzyme activity, yet there will be some loss in digestion efficiency. In order to circumvent this problem, it is recommended to dilute the PCR product minimum 3 folds.

For digesting amplified PCR products, MvaI (or BstNI) restriction enzyme (Roche) was used.

T

5'-C C^A G G-3'

3'-G G T^C C-5'

A

Figure 3.1 Restriction digest sites of MvaI.

The restriction reaction was constructed by adding the reagents in the following order in a total volume of 25µl. The reaction was gently mixed and spun down before being placed in the thermocycler at 37°C for 2 h.

10X Buffer H^+	2.5µl
ddH_2O	39.75µl
DNA	10µl
MvaI (5 units)	0.5µl

c. Clone analysis on agarose gel

1.2% Agarose gels were used to analyze 5-10µl of PCR products, whereas 2.5-3% gels were used to observe RE digestions. The image of each gel was taken under UV light in a BioRad Luminator using Quantity One 4.4.0 software. Clones from each panning were compared to others in terms of size and RE digestion pattern. When the size of a PCR product was equal to another clone, the RE

fingerprint was compared. Representative clones of different sizes and RE fingerprint patterns, together with all undigested clones, were selected for PCR purification and sequencing.

7. Identification of clones

a. Purification of PCR products

After selecting clones for sequencing, 50µl amplified T7 inserts were purified by QIAquick kit (Qiagen) for sequencing. Briefly, 5 volumes of Buffer PB were mixed with 1 volume of the PCR product. The sample was carefully applied on a QIAquick column placed in a 2ml collection tube and centrifuged at 13000 rpm for 30-60 sec. After flow through was discarded and the column was put back into the collector, 750µl Buffer PE (constructed by adding 96-100% ethanol) was added to the column and centrifuged at 13.000 rpm for 30-60 sec. After discarding the flow through, the column was put back into the collector and centrifuged at 13.000 rpm for additional 1 min. Again flow through was discarded and the column was transferred into a clean 1.5ml microcentrifuge tube. 50µl Buffer EB or dd-H_2O was dropped exactly on the center of the membrane and centrifuged at 13.000 rpm for 1 minute. Alternatively, after adding 30µl elution buffer to the center of the membrane, incubation for 1 minute before centrifugation would increase the yield.

b. Sequencing

Purified PCR products were visually quantified on a 1.2% agarose gel using the DNA marker as standard. For PCR products smaller than 1 kb, minimum 50ng DNA and, for longer PCR products, 100ng DNA were sent to an external sequencing service (Sequiserve). Sequences were manually edited by the company and provided both as text file and chromogram.

c. Blast analysis

The insert sequences were analyzed by an NCBI Blast (Balstn and Blastx) search against a non-redundant *Homo Sapiens* database. The identities of each clone were listed. Clones that exist in the list of background clones were omitted. In addition, clones that are common to control samples (epilepsy, NPH, headache, etc) were filtered out from the DS-Ag listing. Remaining clones were evaluated according to several criteria, such as frequency within a certain disease group and within any given patient CSF, and if it existed, the variety of epitopes (different epitopes from the same hit, either overlapping or not).

8. CsCl gradient purification of phage lysate

Larger volumes of phage eluates were purified further using a CsCl gradient.

7ml of BLT 5615 cells were grown in LB buffer with ampicillin O/N at 37°C, shaking at 200 rpm. M9LB media with 20µg/ml ampicillin was made to a final volume of culture 35ml with IPTG added to 1mM, approximately 10 min before infection. The culture was then infected with a single clone and left to stand for a short while on the bench before shaking at 37°C, 250 rpm. After lysis, 3.5ml of 5 M NaCl was added to 35ml culture for centrifugation at 7700 rpm in a Beckman JA-17 rotor at 4°C, for 10 min. 30ml of supernatant was put in a clean 50ml Falcon tube together with 5ml of 50% PEG 8000 and vortexed thoroughly. Next, PEG-lysate was incubated on ice for minimum half an hour or at 4°C for O/N, followed by centrifugation at 7700rpm in a Beckman JA-17 rotor at 4°C, 10 min. Supernatant was removed and the remaining fluid was drained by putting tubes upside down on a paper towel furnished with nylon folio underneath. CsCl gradient was prepared from a stock solution of 62.5% CsCl prepared by adding 25g of CsCl into 15ml ddH_2O. In order to get the gradient we mixed CsCl and TE buffer, starting from the least dense solution, and adding the next denser solution

carefully over it using a pipette. When all the excess liquid had drained, 1.2ml of 5M NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA was mixed with vortex and followed by centrifugation for 10 min at 12000xg. The supernatant was layered onto a CsCl gradient and separated by ultracentrifugation for 1 hr at 35000rpm in an SW41 rotor at RT. Subsequently, the tubes were illuminated from above when other lights were dim, so that the bands became visible. The turbid band above the 2:1 layer was collected and stored at 4°C.

3.2.2 Phage ELISA

Purified phage lysate was diluted 1:20 in 1% BSA-PBS and incubated at 37°C for one hour. Next, stripes from the T7 capture plate were washed with 400µl 0.05% PBS-Tween20. Whenever necessary, CSF was diluted using 1% BSA, and incubated at 4°C, O/N. CSF was washed with washing buffer (400µl 0.05% PBS-Tween20). Subsequently, 100µl of anti-human IgG Biotin antibody cocktail (1:1000 diluted polyclonal (Jackson Imm.) and 1:500 diluted mouse monoclonal from BD Biosciences) was added and incubated for 2 h at RT. After washing the stripes with washing buffer, streptavidin-HRPO diluted 1:2000 in 1% BSA, was added and incubated for 20 min at RT, in the dark and then washed. After adding 100µl TMB (Sigma Aldrich) at RT, the reaction was stopped by adding 100µl of stop solution (Sigma Aldrich) as a differential color development had occurred. The readings were taken at an OD 450 nm with an ELISA reader.

3.2.3 Cloning and expression of rCaspr4 peptide

Cloning, expression and purification of the Caspr4 peptide was performed in collaboration with Dr. Emre Sayan, Leicester University. Briefly, the first coding exon of CASPR4 (genebank id: GI:20544135) gene was amplified by Pfx polymerase (Invitrogen) and cloned into pET/101D-TOPO vector with an N-

terminal 6XHis tag. Three positive clones were selected after sequencing and further analyzed for the expression of recombinant protein. The clone expressing the greatest amount of protein was used for purification. Recombinant proteins were expressed in *E. coli* (BL21) and purified under denaturing conditions using Ni–NTA resin. Proteins were then refolded and buffer exchanged in PBS using NAP buffer exchange columns (GE Amersham). Finally, proteins were concentrated using Centripreps (Millipore). A western blot and Coomassie staining were performed to check the purity, size and amount of the obtained peptide (gel picture is shown in appendix, A3).

3.2.4 RNA Extraction

Freshly obtained mouse brain and spinal cord tissues were frozen and embedded in cryomolds using OCT, and sections were cut with a Cryostat (Leica) and placed immediately on dry ice. For total RNA extraction, the RNeasy kit (Qiagen) was used according to the manufacturer's protocol for frozen tissue sections. 600µl buffer RLT was added onto 10-15 10µm thick sections, and mixed by pipetting. The lysate was transferred to a QIAshredder column (Qiagen) and spun at 14000 rpm for 2 min. at RT for homogenization. After this, samples can be stored at -80°C and should be incubated for 15min at 37°C before proceeding to the next step. 600µl of 70% ethanol was added to the lysate and mixed well by pipetting, 600µl of this mixture was transferred to the RNeasy column and spun at 10000 rpm for 20 seconds at RT. Flow through was discarded and the remaining 600µl was processed identically. 350ml buffer RW1 was added to the column and centrifuged at 10000 rpm for 20 seconds at RT. Flow through was discarded. DNase treatment was performed according to the manufacturer's protocol (Qiagen). Next, the column was washed with 350ml buffer RW1. The collection tube was renewed and 500ml buffer RPE was added before spinning at 10000rpm

for 20seconds at RT. After discarding the flow through, the last step was repeated, but this time spinning for 2 min. The column was transferred to a new Rnase-free 1.5ml tube to elute the RNA with 20-40µl Rnase-free water directly added onto the membrane and spun for 1 minute at 10000rpm at RT. Eluted RNA should be kept at -80°C.

3.2.5 RNA quantification

RNA concentration was determined by measuring the absorption at 260 and 280 nm. The A280/A260 ratio was between 1.8 and 2.0 for all the samples prepared. A ND-1000 spectrophotometer (NanoDrop) was used for quantification of extracted RNA.

3.2.6 Reverse Transcription

A complimentary DNA copy was synthesized using the reverse transcriptase kit (MML-V, Promega). For cDNA synthesis RNase/DNase free filter tips and tubes were used. All solutions required for the process were prepared using molecular grade RNase/DNase free water.

3.2.7 Quantitative PCR (Real Time PCR)

3.2.7.1 Principle

Quantitative PCR was performed on the GeneAmp5700 (Applied Biosystems-ABI) using the qPCR Core kit and UNG (Uracyl-N-glycosylase for carry-over prevention, both from Eurogentec). The reaction volume was 25µl containing 5–50ng RNA converted to cDNA. For detection and quantification of Caspr1, Caspr4, MBP and DDR-1 genes, primers and probes were designed. GAPDH (Glycerinaldehyd-3-phosphat-Dehydrogenase) (Applied Biosystems) was used as

the housekeeping gene (HKG). Percent housekeeping gene values were calculated using the formula: $\%HKG = 100 \times 2^{(-\Delta Ct)}$. Standard deviations of HKG and the gene of interest (GOI) were calculated using the formula based on Muller et al. (2002), given as $SD = 100 \times 2^{(-\Delta Ct)} \times ((\ln 2 \times SD_{HKG})^2 + (\ln 2 \times SD_{GOI})^2)^{1/2}$.

3.2.7.2 Primer / probe design and testing

TaqMan primers and probes were designed using Primer3 (http://frodo.wi.mit.edu/) (Steve Rozen and Helen J. Skaletsky, 2000) and Primer Express v1.0 (Applied Biosystems). All primer/probe sequences were controlled for possible interactions with each other. Designed primers were tested with a conventional PCR experiment using genomic DNA as a template in order to confirm that it was not amplified.

3.2.8 Plaque lift assay

3.2.8.1 Introduction

Plaque lift assay (PLA) is blotting of phage plaques onto a nitrocellulose membrane and then probing with an antibody against the recombinant protein displayed on the phage colony. In this way, it is possible to detect the clones that display the peptide or protein of interest and it additionally helps calculate the enrichment factor. When selecting candidate auto-antibodies against unknown antigens using cDNA PD, it was not possible to perform a PLA as the displayed clone was only identified after sequencing. Additionally, frequently, the antibody against the candidate Ag was not available. Nevertheless, PLA was very useful in the modification of the PD technique during optimizations and pilot selections. For example, monoclonal anti-Synuclein antibody (Sigma, clone 211) was mixed with CSF at variable concentrations binding to the antigen displayed on the phage, and confirmed by sequencing plus PLA (chapter on optimization, section 4.3).

3.2.8.2 Preparations of the nitrocellulose capture filters

Capture antibody was diluted to 10 mg/ml in PBS, pH 7.5. A nitrocellulose filter was incubated with the capture antibody for up to 3 h at RT. The filters were air dried for 10 min and blocked for 2 h at RT with 1% BSA in PBS. Filters were rinsed with PBS and air dried before applying onto the bacterial lawns infected with phage.

3.2.8.3 Plaque lifting

Capture antibody coated filter was applied onto the plaques (maximum 1000 plaques per 100mM diameter plate). After double or triple lifting per plate, the filters were carefully removed, rinsed 4 times with 2ml PBS and blocked for 30 min at RT with 5% skimmed milk in 0.2% Tween20. Primary antibody, diluted in blocking buffer, was incubated for 1hr at RT. Next, the filters were washed with 0.1% PBS-Tween20, 3 times 10 min. Detection antibody was diluted in blocking buffer and incubated for 30 min at RT. ECL plus detection system was used before developing on Kodak film.

3.2.9 Sample preparation for CSF using Vivaspin and Multiple Affinity Removal System (MARS) spin cartridge (optional)

3.2.9.1 Introduction

CSF samples from 11 MDD and 4 NPH patients, assigned for several different proteomics applications, were separated using the Multiple Affinity Removal System (MARS) (Agilent Technologies, USA). After depletion of 6 abundant proteins including IgG, CSF was processed by 2-DE gel electrophoresis and mass spectrometry. The IgG fraction was collected, evaluated for its IgG content and used in panning.

3.2.9.2 Sample (CSF) preparation for MARS spin cartridge

MARS Spin Cartridge has a cartridge capacity of 7-10µl human serum. 18µl serum corresponds to 3ml CSF. Resin bed and frits should always remain moist. MARS spin cartridge has the capacity to process 1.0 - 1.5ml CSF. Nevertheless, the actual cartridge capacity on certificate should be checked beforehand.

3.2.9.3 Vivaspin concentration and buffer exchange

Vivaspin 5kDa MWCO column (Sartorius Biolab) was washed with 2ml ddH_2O with centrifugation at 5000 rpm, RT. CSF was concentrated down to 100µl while flow through was collected and stored at –80 °C. Buffer was exchanged by washing three times with 1ml Buffer A. The flow through was collected and stored at –80 °C. The next step was simply to centrifugation until the final volume was 100 -200µl and filled up to the desired volume with Buffer A. Concentrated product was filtered through a spin filter column (0.22µm) by centrifugation at maximum speed for 1 minute at RT, using a bench top centrifuge.

3.2.9.4 CSF depletion by MARS spin cartridge

Cap and plug of the cartridge were removed. A Luer-Lock adapter was attached to the spin cartridge. 4ml of buffer A was drawn into the syringe ("labeled A") to be attached to the L-L on the cartridge. buffer A was passed through the cartridge to prepare the resin, and excess buffer A was removed from the top of the spin cartridge using a transfer pipette. Next, the spin cartridge was placed in a 1.5ml Eppendorf tube and labeled as FT1 (Flowthrough1). 200µl buffer A was added on top of the resin bed and the open cartridge was centrifuged at 80rcf for 1.5 min (the flow-rate was measured beforehand and ideally has to be ~0.2ml/min). 400µl Buffer A was added on top and centrifuged at 80rcf for 2.5 min. FT was collected into tube FT1 and the spin column was placed in a new collection tube, labeled FT2 (Flowthrough2). 400µl Buffer A was added and centrifuged at 80rcf for 3 min. The spin cartridge was removed from tube FT2 and the L-L adapter was attached to the top. A 5ml L-L syringe was filled with 2ml buffer B and the spin cartridge was attached via the L-L adapter. IgG and other high abundant proteins were eluted into 1.5ml eppendorf tubes and labeled as eluate by slowly pushing 2ml buffer B through the spin cartridge. In order to re-equilibrate, a 5ml syringe was filled with 4ml buffer A, and pushed slowly through the spin cartridge. For storage, a layer of buffer A was left on top. Both ends were recapped, and the cartridge is stored at 4°C.

FT2 fraction usually contains less than 10 % of the total FT. If necessary, FT1 and FT2 fractions can be combined and concentrated again. For my purposes, I checked the IgG quantity that might have leaked into FT. In order to analyze the eluate, one may have to perform a buffer exchange to PBS, or another buffer compatible with the analysis as buffer B may interfere with some protein assays.

3.2.10 Human IgG ELISA quantification

After the Vivaspin-MARS procedure, the samples were evaluated by an IgG Elisa quantification kit (Bethyl Laboratories). All steps were performed at RT and all samples were analyzed in duplicate. Briefly, 1µl of monoclonal anti-Synuclein clone211 antibody (Sigma) was mixed with 100µl coating buffer and incubated for 60 min in ELISA wells. After washing 3 times, the wells were blocked with 200µl blocking buffer for 30 min. 100µl volumes of standards and samples were prepared in the dilution buffer and incubated for 60 min. After washing 5 times, 100µl of anti-mouse-HRP conjugated detection antibody diluted 1:50000, was applied to each well and incubated for 60 min. After washing, TMB substrate (Sigma Aldrich) was used for detection and the reaction was stopped using sigma stop solution before reading at 450nm by an Elisa reader.

3.2.11 Western blot

For qualitative analysis, samples were loaded directly without measuring the protein content, whereas for quantitative analysis, protein concentration was measured by BCA protein assay (Pierce).

Protein samples were dissolved in equal volumes of 2x Laemmli sample buffer and heated at 95°C for 5 min. Samples were spun down shortly before loading onto a 12-15% SDS-PAGE gel. After transferring onto a nitrocellulose membrane, the membrane was treated with Ponceau Red (Pierce) before blocking for 1 hour at RT. Primary antibody was diluted in blocking buffer and incubated at 4°C O/N. After washing the membranes 3 times for 10 min, secondary Ab-conjugate in blocking buffer was applied and incubated for 1 hour at RT. After washing, appropriate chemiluminescence substrates were added (ECL for HRPO conjugates and CDP-Star (Novagen) or for AP substrates). Signals were developed with a Kodak film. Alternatively chromogenic detection of alkaline

phosphatase conjugates was developed on the membrane by a purple precipitation using NBT/BCIP substrate.

3.2.12 Protocol for control samples used in optimization

For initial screening of depression-related IgG repertoire, 2 unipolar depression (UPD) and 2 normal pressure hydrocephalus CSF samples were used. The secondary aim was to characterize the CSF background and to compare MARS depleted and non-depleted CSF samples. Samples MDD 312 and NPH 2 were processed by the MARS spin cartridge so that depleted IgG fraction from CSF was introduced into the panning. Samples Lö and NPH3 were used directly from the original aliquot. The new IgG concentration was measured by the Human IgG ELISA kit. Eventually, 5µl of IgG per panning was immobilized on Dynal tosyl-activated magnetic beads coupled to anti-human IgG antibody specific to the Fc region. During panning steps, washing was carried out by 1% TBS-T with increments in duration for each round. Elution was performed with 5M NaCl for 25 min at RT. Eluates from the last panning round were plated on agar bacterial loan and 24 plaques were picked from each sample for clone analysis.

Chapter 4

cDNA Phage Display, CSF and Autoantigen Research: Challenges and Optimizations Addressing a Novel Approach

4.1 Introduction

Peptides and proteins can be displayed on phage particles for autoantigen selection from human material. Nonetheless, there are major factors that can greatly affect the overall success.

Most importantly, the cDNA library to be implemented within the PD selection system must bear a sufficient number of recombinant clones in the range of a minimum of several millions. Secondly, the whole system with regarding to the type of display (monovalent, polyvalent), type of phage (filamentous, lambda, T7) has to be researched and tested for suitability. Finally, stringency must be fine-tuned for selecting mostly the true hits with a minimum of false positives. Actually, co-elution of sticky and false positive clones is a constant drawback, which can be improved upon but never totally eliminated. Only after a series of stringent control steps, can there be any confidence in the success of selection as there is very limited sensitivity in ruling out false positives from true hits.

CSF is a valuable human material collected in rather small volumes through an invasive technique with almost no sampling from healthy subjects. Similar to many other studies, CSF was the limiting factor in this work as well.

Psychiatric disorders, like BPD and SZ, have been studied thoroughly by investigators for an underlying autoimmune condition. It is still a challenging task to find autoantigenic targets that are not as evident or dominant as in case for established autoimmune disorders.

Referring to all the issues mentioned above, this thesis was based on an exploratory project that requires not only literature-based, but also original strategies that address the feasibility of our approach to unravel minute autoimmune activities for psychiatric disorders.

This chapter summarizes the development of our exploratory approach, elucidates strategically important decisions that were taken and critical parameters and explains the modifications made for the standard PD protocol in order to obtain adequate sensitivity and a relatively low false positive background, while surmounting the limitations of CSF. Results included herein demonstrate the principle and the outlook of the technique, thus providing supportive evidence of successful applications. Subsequently, obtained results were evaluated stringently and the most promising candidate autoantigen was selected for further analysis.

4.2 Important parameters of a phage display experiment

4.2.1 Type of display

Peptide libraries are generated as *in vitro* synthesized, randomly expressed as N-terminal fusion to minor coat protein pIII (monovalent display) or major coat protein pVIII (polyvalent display) on filamentous phage. Peptide PD libraries were used especially for studying the binding properties of homogenous proteins. Nevertheless, there are many reports about finding autoantigens from sera of autoimmunity disorders like rheumatoid arthritis (Dybwad et al., 1995, 1996; Hansen et al., 2000) multiple sclerosis (Rand et al., 1998; Cortese et al., 1998; and systemic lupus erythematosus (Dieker et al., 2005). Similarly, cDNA PD has been successfully applied in autoantibody searches in polyclonal human material like sera of rheumatoid arthritis (Mewar et al., 2005) and systemic lupus erythematosus (Kemp EH et al., 2002) patients.

Epitopes from processed antigens, presented as peptides by MHC class I and II, can be studied by peptide PD (Sioud, Hansen and Dybwad, 2000). Such applications include elucidation of target immunogenic epitopes for multiple sclerosis (Cortese et al., 1996; Rand et al., 1998).

In addition to processed antigens, cDNA PD also enables identification of parental antigens that are targets of B-cells, bearing bound or secreted immunoglobin (Ig) molecules that are receptors of soluble or pathogen-bound antigens (Sioud, Hansen and Dybwad, 2000).

For our query on psychiatric disorders, of which the current knowledge in disease etiology is not based on a substantial autoimmune attack, the primal idea was to see whether autoantigenic molecules could be detected at all. For this initial step, identification of the autoantigenic target was sufficient. Accordingly, for this application, the cDNA PD system is more powerful than the random peptide display as the latter has the capacity to display only a small fraction of all possible sequences. In contrast, the cDNA library represents all the mRNA expression from a tissue at the period of extraction. Despite the existence of 3'UTR inserts and unknown gene products that cannot be excluded, cDNA libraries represent mostly the naturally expressed antigens, whereas peptide libraries include several unnatural epitopes (Sioud, Hansen and Dybwad, 2000). The cDNA product that is likely to be a candidate autoantigen is identified by sequencing, thus the information on the epitope mapping of the interaction is simultaneously obtained.

As a result, the cDNA PD library strategy was chosen as it has potential to reveal autoantigenic targets within the CSF of the psychiatric disorders in question.

4.2.2 Quality of the library

The most important parameter of a successful PD project is the quality of the initial library, reflected in the diversity of the recombinant clones. Normally the range is between 10^6 and 10^{10} plaque forming unit (pfu) recombinant clones.

The diversity of the self-constructed or commercially obtained library should be tested before any application. It should always be kept in mind that all recombinant clones are not equally expressed within a library. For example, abundant proteins of a tissue are reflected as abundant transcripts within cDNA libraries. Similarly, as seen in peptide libraries, some sequences may be toxic, difficult to display due to rare residues or not compatible with the phage itself (Smith and Petrenko, 1997).

The T7 Select library used in this study encodes cDNA obtained form normal brain with $1.47x10^7$ recombinant clones. The insert size is between 0.3 and 3kb after PCR analysis of the amplified library.

Maintenance of the library is vital and the library should be kept in aliquots, as repeated freeze-thaw cycles will reduce the titer.

A further point is about the number of propagations that can be applied on the original library without losing its diversity. Generally, it is advisable not to propagate the library more than twice. In fact, the risk of losing under-represented clones at this very early stage is so high that it could easily jeopardize the whole selection from the beginning. The T7 select library was amplified in liquid culture only once before the whole selection was performed.

4.2.3 Stringency versus yield

During each panning round, stringency regulates the threshold for selecting better binders against non-specific ones. Thus, the stringency level determines the yield. A convenient PD library has a minimum of 10^6 recombinant clones with 100 copies from each clone.

The first round selection is of utmost importance because if none of the, for example, 100 copies of a given clone are able to bind to their targets due to excessive stringent conditions, that clone is lost and cannot be regained. Accordingly, yield is always favored over stringency for the first round of selection. Already after the first amplification step, the first round binders are represented in millions of copies, in other words, they are enriched against less specific binders, enabling re-adjustment of stringency. However, it is not advisable to practice extreme stringency in the following rounds, as it would dramatically reduce the yield. Moreover, it may push forward the non-specific but persistent clones that are

better survivors than high affinity binders, eventually causing the sticky background to outnumber the true hits (Smith and Petrenko, 1997; Russel, Lowman, Clackson, 2004). A balanced fine-tuning between stringency and yield is only valid after several tests of various conditions and modifications. When exploring the feasibility of the T7 system, efficiency of the selection conditions were tested by means of pilot experiments given below (see 4.3.1 - 4.3.2). Subsequently, the sensitivity of selection and efficiency of elution were optimized (4.3.3-4.3.6).

4.2.4 Selection

In general, the target is tethered to a solid support like a microtiter plate, magnetic beads or porous agarose beads. Microtiter plates are practical to use and compatible with multi-channel pipettes but provide a limited area for the target to bind. By comparison, beads offer a maximum surface area and usually have better washing efficiency. However, due to handling mistakes, some beads may get lost during washing or elution, plus extensive contamination can arise. The tests regarding the magnetic beads used in this study seek to understand the limits of BioMag beads preserve CSF-IgG binding without covalent attachment (see 4.3.2.2).

Another critical consideration is the number of rounds for selection. Enrichment is the key parameter to monitor if selection is working at all, and when to stop panning. To pursue panning after stabilization of enrichment endangers potential true affinity clones that are not as efficiently propagating as the others.

An option, which was applied in this work, is to run a pilot selection with a known target to test the typical enrichment pattern of the library, and conditions for selection, and to see when to stop in a relatively similar set up (section E and F).

4.2.5 Analysis of the clones

In general, the clones selected from the last round of panning were analyzed individually. A single plaque on a bacterial lawn represents 10^6-10^7 phages from the same genomic construct. DNA purification and a subsequent sequencing provide the identity of the clone and the nucleotide sequence of the insert within the phage genome corresponding to the amino acid sequence. If necessary, the next step is to clone the insert into an expression vector for further analysis (Smith and Petrenko, 1997). In fact, one clone, identified in this study, was cloned to generate recombinant protein to be used in an Elisa.

4.3 Results

4.3.1 Feasibility of the T7 system:

4.3.1.1 Standard screening conditions applied to the T7 phage display system yield significant enrichment after 4 rounds of panning

For evaluating the feasibility of the T7 Select system, the strong binding interaction between the 15 amino acids long S-tag and the 103 amino acids long S-protein, both derived from Rnase A, was employed (Figure 4.1). T7 Select positive phages bear the T7Select415-1 vector with the S-Tag insert, whereas the negative control vector has no insert.

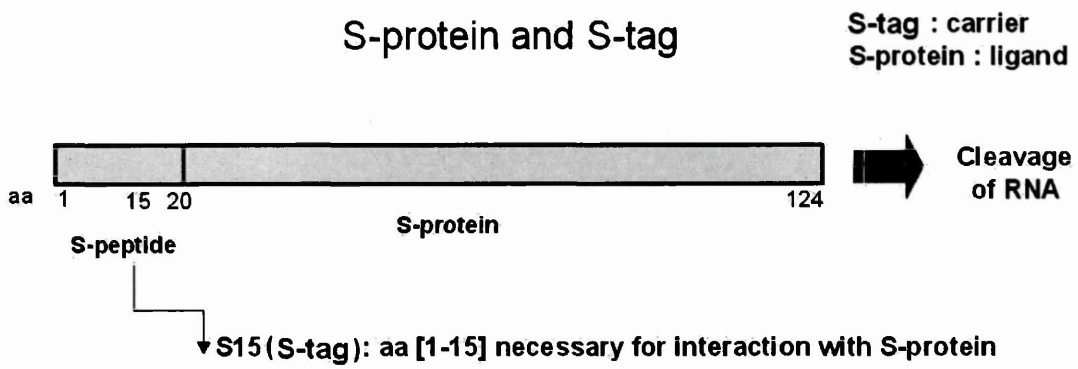


Figure 4.1 S-Tag peptide is displayed on the surface of the T7 phage as an in-frame fusion protein. The insert has been cloned into EcoR I/Hind III arms of T7Select415-1 vector.

S-protein was coated in the wells of microtiter plates (Corning HighBind) at 1μg/ml to 10μg/ml concentrations. In parallel, Dynal M-280 tosyl-activated magnetic beads were covalently coated with S-protein (0.7 μg/20μl beads). A phage library with a ratio of positive to negative clones of 1: 10^6 was used for the screen (input). After 4 rounds of panning enrichment of the S-tag insert-carrying phage was assessed by counting the number of positive clones using a standard plaque lift assay (Figure. 4.2). When microtiter plates were used as a matrix, a maximum

1.25 x 10^5 enrichment of S-tag phage was found, whereas 2.8 x 10^4 enrichment was observed after the magnetic bead immobilization procedure (Table 4.1).

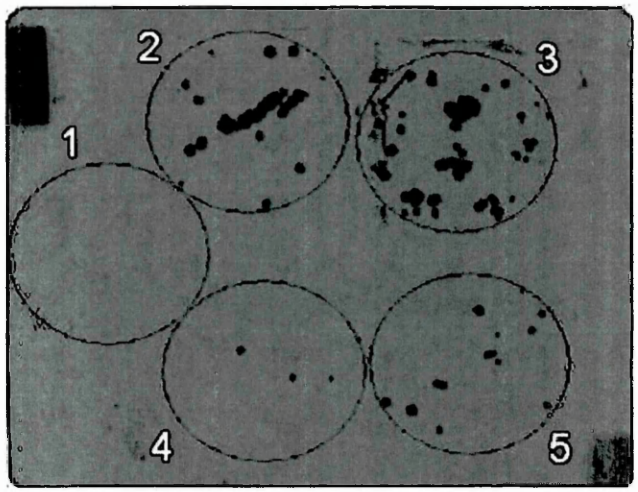


Figure 4.2 Plaque lift assay results (standard selection conditions). Four rounds of panning were performed on S-protein. Non-specific binders were washed with 0.1% TBS-Tween20 for 3 times 10 min each. 5M NaCl was used for elution at RT for 25 min. The plaque lift assay was performed using S protein HRPO conjugate at 1: 5000 dilution. The film was overdeveloped to show the smallest plaques, yet the counting was taken when the plaques were well-separated.

Table 4.1 Comparison of enrichment. Titer assay, performed after the last round, facilitated evaluation of the enrichment factor.

	Panning Procedure		Plaque Count		Enrichment
	Target	Matrix	**Total**	**Positive**	
1	Non-coated	Microtiter	500	0	-
2	S-protein (1 μg/ml)	Microtiter	400	30	$7.5x10^4$
3	S-protein (10 μg/ml	Microtiter	400	50	$1.25x10^5$
4	BSA (1 μg/ml)	Microtiter	500	3	$6x10^3$
5	S-protein (0.7 μg/ml))	DynaBead	500	14	$2.8x10^4$

4.3.1.2 Adjusted stringent selection conditions improve signal to noise ratio without decreasing the yield

S-protein positive lysate was mixed with negative lysate ($1:10^6$), as well as T7 whole human brain cDNA library composed of $1.47x10^7$ recombinant clones, again with the ratio of positive clones to recombinants as $1:10^6$. Increments in stringency were devised after each round with longer washing durations (three times, up to 30 min each). Increased Tween-20 concentrations of washing buffers were tested and up to 0.2% PBS-Tween-20 was applied during washings during the third round. Background was measured by panning on non-coated wells. Positive clones were identified by plaque lift assay after 4 rounds of panning. Figure 4.3A shows the detection of S-tag insert clones with no background clones found for the negative control. Same strategy was repeated using whole library instead of negative lysate (Figure 4.3B)

A B

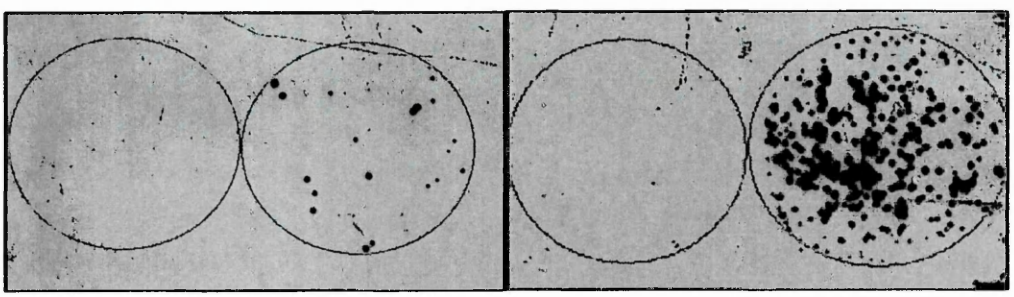


Figure 4.3 Plaque lift assay results (stringent screening conditions). T7Select Brain library consists of 1.47×10^7 primary recombinants. All membranes were probed with S protein-HRPO conjugate. **A. S-tag positive versus negative lysate**. The left membrane shows control panning on non-coated microtiter wells, whereas the right membrane shows the panning carried on microtiter wells coated with 100 μL of 0.5 μg/ml S-protein. Enrichment was found to be 8.3×10^4 .
B. S-tag positive versus whole library. The membrane on the left shows control panning on non-coated microtiter wells and the membrane on the right shows S-tag displaying plaques obtained from last panning round on 5 μg/ml S-protein (10 fold more target compared to panel A) coated wells.

4.3.1.3 Sensitivity of T7 system is optimized and tested using anti-synuclein antibody as the known antibody target

Anti-synuclein mouse ascites (Syn-211; generated against amino acids 121-125 of the protein, Sigma Aldrich) were coated with an approximate Ig concentration of 1, 2 and 10 μg/ml in TBS on HighBind Corning microtiter strip-wells. After 4 rounds of panning, enrichment was assessed by titer and plaque lift assays. No background was observed for the negative control (panning on non-coated wells).

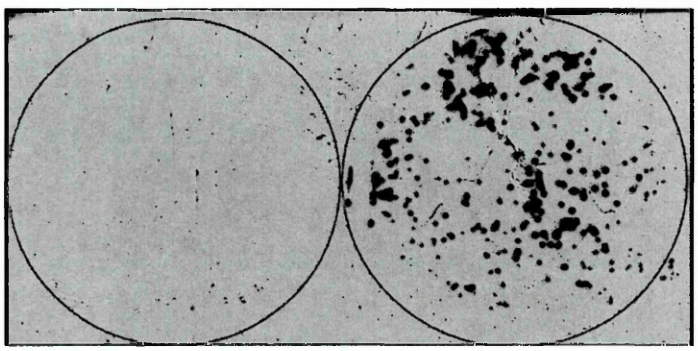


Figure 4.4 Plaque lift assay conducted after enrichment with anti-synuclein antibody. Left filter: control (no antibody); right filter: anti-synuclein ascites, dilution of anti-mouse HRPO is 1:2000.

Optimizations were based on alternative immobilization techniques. Instead of direct immobilization of the target on microtiter wells or Dynal beads, solution binding of the target and phage was tested. Binding in solution, allows phage to interact with the target freely in solution thus reducing avidity effects. The beads subsequently capture phage and target, as a complex. Magnetic beads with a 280 mm diameter were covalently coated with recombinant protein G (Dynal) and prepared according to the manufacturer's protocol. 10ng of anti-synuclein antibody was incubated with the brain phage library and the resulting complex was captured with protein G magnetic beads. PCR was carried out using the whole lysate, as well as individual plaques. Clones were analyzed by agarose gel electrophoresis and prepared for sequencing. PCR of the whole lysate from the last panning yielded 2 major bands that were 700 bp and 850 bp in size (data not

shown), where the insert with 700bp size was identified as alpha-synuclein, but the 850 bp clone was unrelated (background). Plaque lift assay was performed to detect synuclein displaying clones (Figure 4.5).

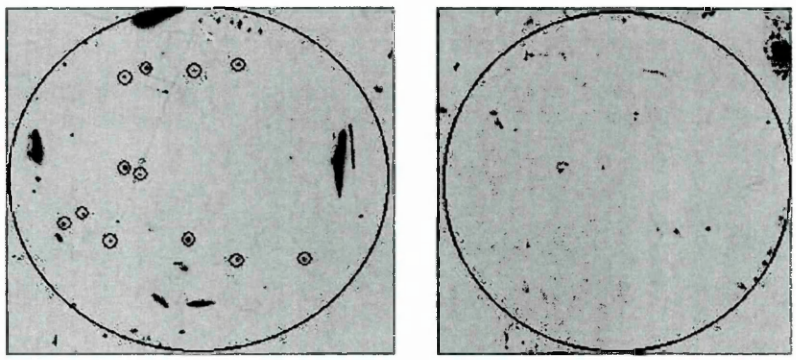


Figure 4.5 Synuclein positive clones. The membrane on the left depicts plaque lift assay results using Syn-211 antibody. After plating the lysate from the last panning, plaque lifts were performed using nitrocellulose membranes in disk format (3M) and blocked for 2 h. The membranes were incubated with anti-Syn antibody (Sigma) at 1:2000 dilution and detected by anti-mouse HRPO (Sigma) at 1:2000 dilution and ECL system. The spots encircled in red are anti-Syn reactive phage lysates. The same lysate on the right membrane, treated only with secondary antibody, did not yield any signal.

4.3.1.4 T7 compatible bacterial strain BLT 5615 generates less background compared to BLT 5403 strain

While monitoring the efficiency of the T7 system, *E.coli* strain BLT5403 was found to generate a higher background compared to BLTR5615, mainly in plaque lift assays. PCR analysis of the plaques showed that BLT5403 produced shorter inserts than expected (data not shown). In the manufacturer's manual, strain BLT5403 is described to be slightly more prone to deletion mutations in the capsid protein if the inserts are bigger than 600 bp (Novagen). In addition, BLT 5403 plaques looked smaller than BLT 5615 plaques when plated on LB-agar, although the titers were equivalent.

In conclusion, propagation of eluted phages using BLT5403 was unsuccessful Instead; IPTG inducible BLT 5615 *E.coli* host seems to be a better option for amplification. PCR analysis of selected clones propagated by BLT5615 culture confirmed regular insert sizes that range between 300 bp-3 kb (data not shown). Based on these results, panning was performed from BLT 5615 lysates.

4.3.1.5 Monitoring elution efficiency

Elution must be rigorous enough to collect almost all of the selected clones. The clones with lower dissociation rates might not co-elute and stay on the matrix. In fact, those clones that bind with high affinity to the matrix are of utmost interest and must be efficiently recovered.

Antibody-antigen interaction is rather efficient in aqueous buffers at physiological pH and ionic strength. Thus, elution can be accomplished by altering the pH or the ionic state. Modified PD protocols were tested for elution efficiency in terms of alternative elution buffers (5M NaCl versus 1% SDS) and incubation times recommended for T7phage. The efficiency of elution was assessed by T7 phage ELISA that measures phage particles that are still "binding" to the matrix, and also by a titer assay that measures rescued phages after the elution step.

Standard tests showed efficient elution using 5M NaCl buffer at 25 min of incubation with small-scale tests with known targets. For comparison, a longer incubation for 35 min was tested. Results implicated no significant increase of the elution efficiency due to duration of elution and/or change of buffer. Nevertheless, one difference between test conditions was that the 5M NaCl eluate could be directly added to bacteria, whereas SDS had to be diluted to prevent bacteria from dying before efficiently producing the phage particles.

4.3.2 Optimal utilization of CSF in phage display selection

4.3.2.1 Strong binder phage subtypes with low dissociation rates can be rescued from the beads by an *in-situ* infection step

Elution buffers and durations were quite efficient but the test results did not reveal on how to rescue strong binding clones with slower dissociation rates.

As the limiting factor was CSF-enriched IgG, any phage capturing CSF-IgG via its displayed fusion peptide is very important for the success of the selection. In order to make sure that almost all of the selected high affinity clones were rescued, an additional step was introduced to the first and second panning rounds, namely, *in situ* infection of bacteria directly on the beads.

In situ infection was initially described by Wind et al. in 1997 and was previously used in finding receptors for orphan ligands (Wojnar et al., 2001) and in the generation of antibodies (Krebs et al., 2001), as an attempt not to jeopardize high affinity binders.

Briefly, an aliquot of the bacterial culture at mid-log growth phase was infected with the phages that presumably still resided on the beads after the standard elution had taken place. As the phages are bound to the target by the peptide as fusion to capsid, the tail proteins are free to attach bacterial receptors, rendering them capable to infect further. After allowing a minimum 15 min for infection, supernatants from the beads were transferred to culture that had been infected previously with eluates and was propagated with good aeration and vigorous shaking.

4.3.2.2 CSF IgG can be displayed as IgG-constant domain (Fc) fusion using BioMag beads

Another important factor is the efficiency of the beads used as the matrix to preserve the captured CSF-IgG throughout the incubation and washing steps. As an alternative to Dynal beads M-280, BioMag beads (Qiagen, Polysciences) were tested. BioMag beads are available covalently coated with anti-human IgG Fc antibodies. Different binding and washing conditions, and the ability of BioMag beads to keep IgG bound to the surface, were tested.

Fluorescence-labeled human IgG whole molecules (hIgG-FITC), captured on BioMag-Fc specific beads, were measured by Fluorescence Activated Cell Sorting (FACS) analysis. Counting FITC labeled-immunoglobulin molecules within the supernatants, collected after binding and washing steps, helped evaluation of unbound or released IgG particles. By the same principle, IgG in supernatants was quantified by ELISA.

Both results yielded a satisfactory percentage of binding at 4°C after 6hrs of incubation (500μl beads capturing up to 10μg IgG). Only negligible amounts of bound IgG were detached at adjusted washing stringency.

In this respect, BioMag beads were used to reproduce results from a panning performed with Dynal beads. Eventually, preparation of the BioMag beads was much faster compared to the Dynal beads that required 24hrs incubation for covalent binding of anti-human IgGs before coating with CSF-IgG. Additionally, BioMag beads were more economical when excess antibody, used to ascertain adequate coating of the Dynal beads, was taken into account. Panning performed by both types of beads resulted in a similar enrichment quality and yielded a

number of common hits, implicating a comparable efficiency. As a result, BioMag-human IgG Fc specific beads were preferred for immobilization.

4.3.3 Feasibility of consumption of a CSF sample in multiple proteomics applications

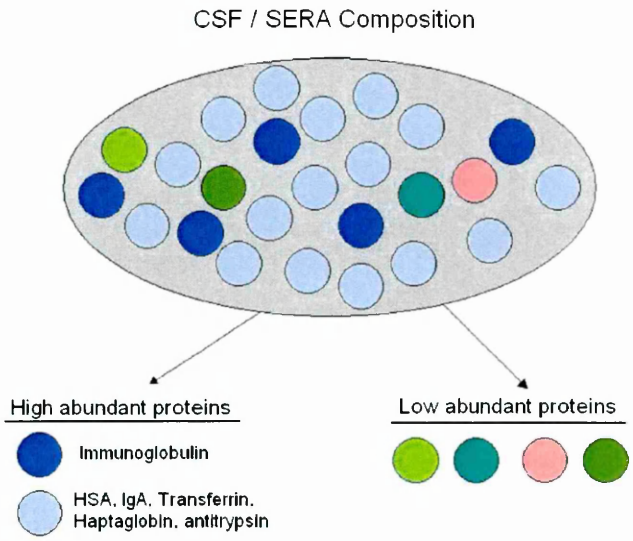
A parallel objective during empirical optimizations was to investigate the likelihood of a better usage of CSF allowing several proteomics projects to share CSF samples.

The standard proteomics biomarker platform involves the depletion of the six most abundant proteins, including IgG, from CSF samples by an immunoaffinity separation system. If successful, this manipulation allows sharing of the same CSF sample in multiple proteomics approaches. In order to ascertain that immunoglobulins were not lost, and the binding affinity to their respective brain antigens not compromised, test antibodies mixed with CSF were initially processed by immunoseparation and then used as targets for the panning.

For this purpose, separation (depletion of abundant proteins from crude CSF) was performed by an immunoaffinity chromatography called Multiple Affinity Removal System (MARS) (Agilent Technologies, USA) (Figure 4.6).

The feasibility of this approach was questioned to see whether this manipulation would alter sample quantity and/or quality for panning.

A



B

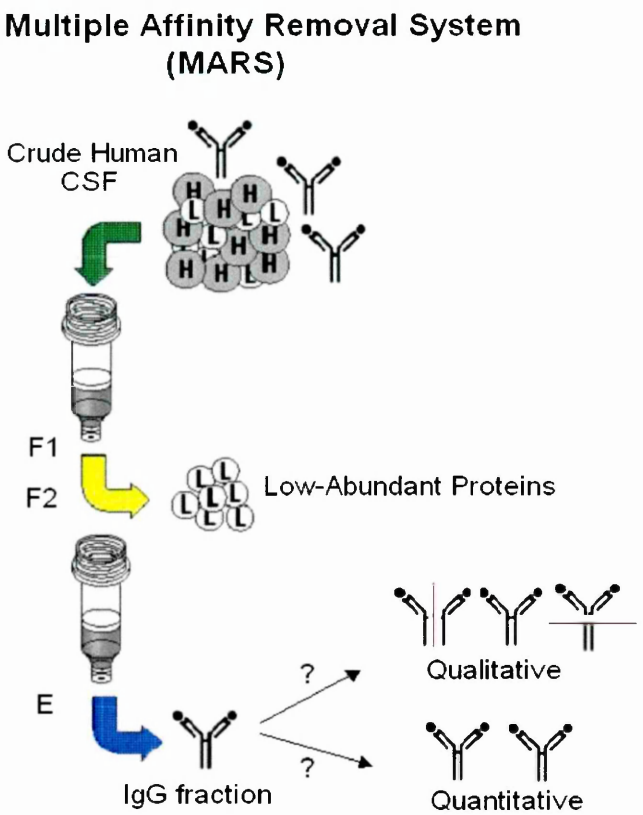


Figure 4.6 Principle of MARS immunoaffinity spin column. A. High abundant proteins are depleted from sera or CSF by immunoaffinity polyclonal antibodies. **B**. Crude CSF corresponding to a maximum of 3 mg of total serum protein may be processed by spin cartridge without clogging. Flow through is mainly composed of low abundant proteins. Eluate is a collection of albumin, IgG and IgA, transferrin, haptaglobin and antitrypsin that dissociate from the matrix after treatment with a buffer at pH 2.3 with 2M Urea. Illustration A was prepared by E. Sayan-Ayata, illustration B was modified from the manual of the MARS spin cartridge.

4.3.3.1 CSF IgG fraction isolated by MARS immuno-affinity spin column can be successfully applied as target in panning

A low pH buffer with 2M urea was used for elution of abundant proteins that were affinity-captured by the MARS spin cartridge system. Thus, the integrity of Igs after the elution step needed to be tested. First, a given amount of a known antibody was added to the CSF sample which had passed through the Vivaspin concentration/buffer exchange step and been processed by the MARS spin cartridge. Next, a standard biopanning experiment was performed to see if it was possible to capture MARS processed IgG molecules as targets. For this purpose, the hydrocephalus CSF sample was processed with the MARS spin cartridge. Control Ab was added (α-synuclein mAb, clone 211, Sigma Aldrich) in varying concentrations of buffer B, followed by Vivaspin buffer exchange to PBS. Later, the target was immobilized on Protein G beads and panned using the 50% α-synuclein enriched sub-library (own preparation). Table 4.2 shows experimental setup. After panning, the titer of the eluate was measured to investigate the binding affinity. Additionally, plaque lift assay was performed (Figure 4.7) and enrichment was measured (Table 4.3). Results showed that if the CSF sample was immediately neutralized after MARS separation, IgG was recovered and detection of the anti-synuclein antibody within the CSF IgG pool could be reproduced.

Table 4.2 Experimental setup for panning of MARS spin column processed samples

	CSF	BUFFER B	PBS	SYN 211 antibody	α-SYN pos/neg library
1	-	-	+	1250ng	+
2	-	+	-	1250ng	+
3	-	-	+	-	+
4	+	+	+	-	+
5	+	+	+	50ng	+
6	+	+	+	250ng	+
7	+	+	+	1250ng	+

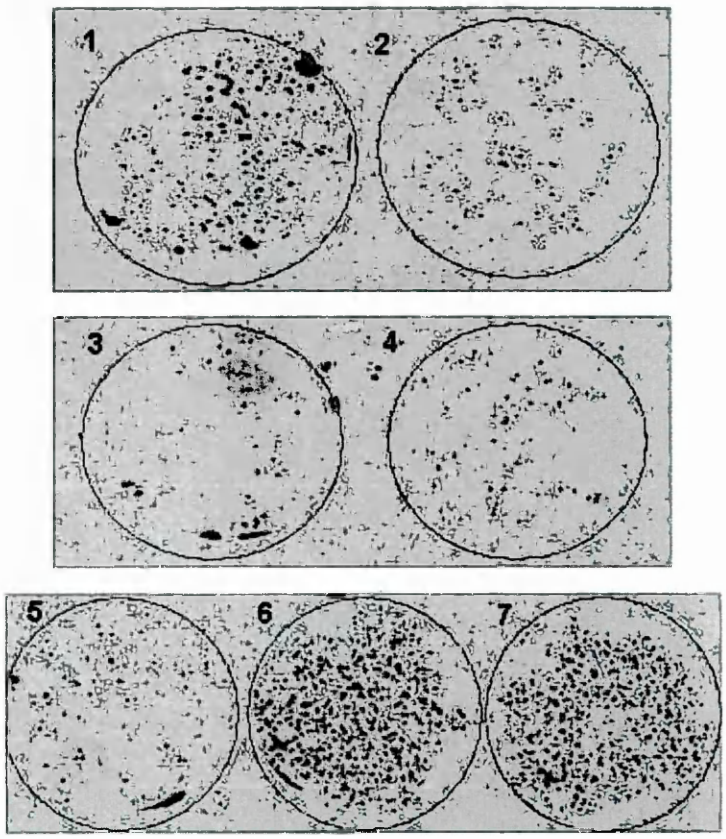


Figure 4.7 Results of test panning after MARS spin column. Anti-synuclein mAb is used as a target for panning with the T7 normal brain PD library. 1250ng α-synuclein preparation panned without buffer exchange (sample#2) had partially lost its ability for biopanning detection, although the same amount of antibody mixed with PBS reached 50% enrichment. Samples panned after buffer exchange to PBS (sample#1, #5, #6, #7) recovered a gradual panning efficiency, as observed by the enrichment according to the synuclein input amount.

Table 4.3 Titer assay after the last panning of MARS spin cartridge processed samples

Controls	Titer (pfu/ml)	Total	Positive clones	Enrichment
Input		2	1	
Output #1	4×10^3	507	240	Saturation (~1)
Output #2	2×10^3	444	50	0.2
Output #3	$\leq 10^2$	150	20	Background (13%)
Output #4	$\leq 10^2$	590	30	Background (5 %)
Output #5	2×10^4	578	110	0.4
Output #6	2.5×10^4	1100	630	Enhancement (1.2)
Output #7	3×10^4	1000	530	Saturation (~1)

4.3.3.2 Test IgG sample was at least partially deteriorated in terms of quality and quantity after MARS-HPLC fractionation of CSF For analytical grade experiments, high performance liquid chromatography (HPLC) separation is feasible. Hence, the MARS HPLC column was compared to the MARS spin cartridge system regarding applicability.

During MARS-HPLC separation, exposure to the relatively harsh elution buffer (low pH with 2M urea) is much longer than in the case of the spin cartridge system. As a result, it was uncertain whether IgGs were irreversibly affected. In order to minimize degeneration of IgG, the MARS HPLC protocol was modified so that all the steps (flow-through, elution and equilibration) were held at 4°C, plus, the sample was immediately manually collected into a neutralizing buffer, just before elution started, and until equilibration peaks were observed.

As a target, anti-synuclein antibody was processed with Vivaspin followed by MARS-HPLC, and subsequently quantified with ELISA before panning. Table 4.4 shows experimental setup.

Unfortunately, a clear decline in the amount or functionality of IgG was obvious when results were compared to data from the spin cartridge application (Figure 4.8A).

Furthermore, a mAb (anti β-actin) was tested on A431 cell lysates with western blot to check the quality of the antibody after MARS-HPLC processing. Anti β-actin lost its avidity almost completely, as seen in western blot (Figure 4.8B).

Eventually, despite all the efforts described above, the IgG fraction seemed to be affected quantitatively and qualitatively after HPLC processing.

Table 4.4 Experimental setup for panning of MARS HPLC processed samples

	CSF	BUFFER B	PBS	SYN 211 antibody	α-SYN pos/neg library
1	-	-	+	1000ng	+
2	-	+	-	1000ng	+
3	-	-	+	-	+
4	+	+	+	-	+
5	+	+	+	50ng	+
6	+	+	+	100ng	+
7	+	+	+	250ng	+

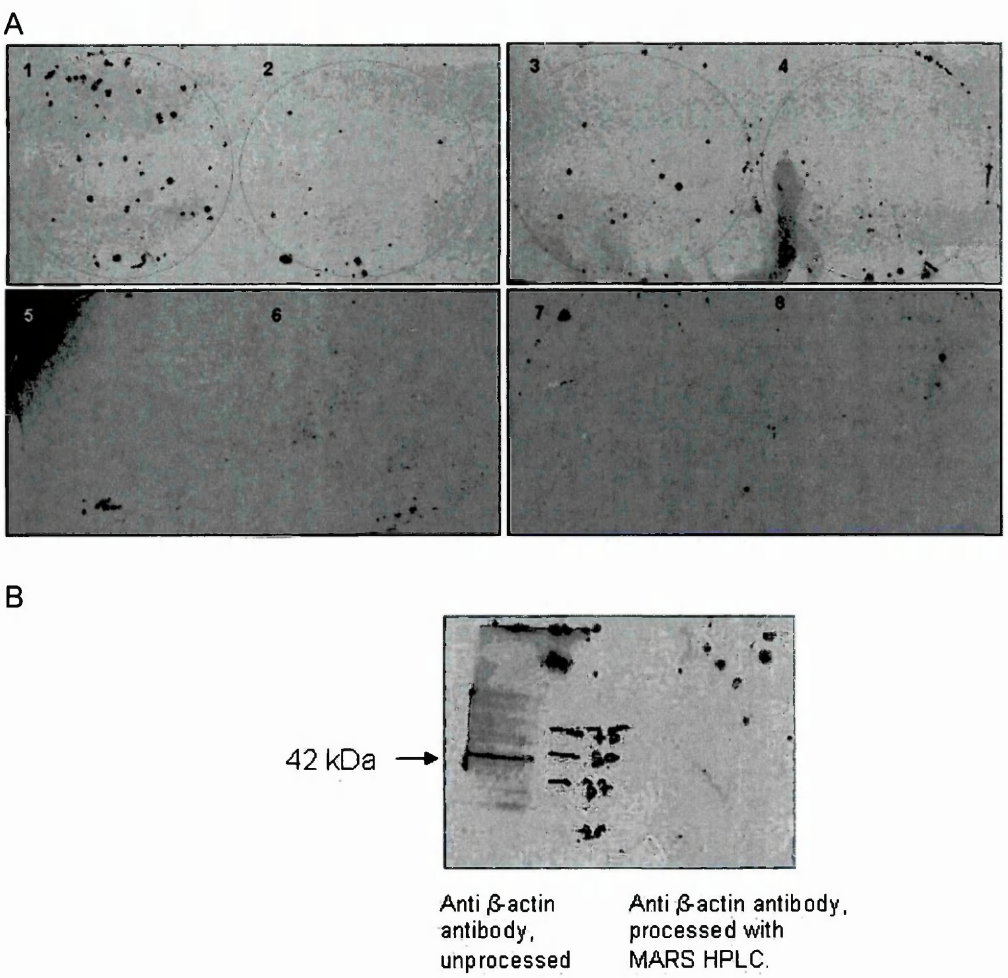


Figure 4.8 Results of test panning after MARS HPLC. Synuclein monoclonal antibody is used as a target.1 μg α-Synuclein preparation panned without buffer exchange (sample#2) had lost its ability for biopanning detection, although the same amount of antibody mixed with PBS was detectable (sample#1). However, it was still not possible to detect the samples after buffer exchange to PBS (sample #5, #6, #7) (A). Anti β-actin antibody was processed by MARS spin cartridge and used as primary antibody in western blot on A431 cell lysates. Positive signal at 42-kDa molecular weight was observed when original β-actin antibody was used at a dilution of 1:1000. There was no signal with MARS-HPLC processed anti-β actin antibody used at 1:1000 dilution after 15 min of overexposure (B). However, a very weak signal could be obtained when the film was left for overnight exposure (not shown).

4.4 Strategies for filtering false positives

A constant drawback of PD is the accumulation of false positive clones over succeeding selection rounds. This is common to all PD systems, independent of the type of phage (filamentous, lambda or T7) and type of library (peptide, cDNA, IgG-Fc) (Kramer et al., 2003). They are not easy to distinguish and eliminate. Background clones and false positives may result from selection conditions, molecules involved in the selection, inefficient blocking or due to affinity for the solid support. Even at the most optimized stringency, sticky clones may get enriched up round after round to a level where they can completely mask specific clones of interaction.

The most common way to evaluate the selection is via monitoring the enrichment. There are two types of enrichments: the background enrichment and the marker enrichment. In order to monitor the marker enrichment, phage vector (phagemid) has to encode a selective marker like a reporter (β-galactosidase) or a drug resistance. For instance, eluted phages can be plated on an IPTG/Xgal plate and the blue/white ratio will give the specific interaction versus binding to non-coated / affinity matrix. Employing a negative control (empty phage) can help monitor the titers of the background to help compare the enrichment of the affinity selection (Russel, Lowman, Clackson, 2004).

One strategy, applied in this study, was to reduce the background by pre-clarification of the cDNA phage library used in the panning. More specifically, normal brain cDNA library was incubated with excess BioMag anti-human IgG-Fc beads, so that the phages that would have affinity to the matrix (BioMag beads)

were isolated. The recovered library was theoretically free of the BioMag reactive clones, thus adding straightforwardness to the protocol.

Another control, introduced in this project, was monitoring the marker enrichment by an alternative panning using serum in parallel to CSF. While IgG from patient CSF was under selection against the cDNA brain library, total human serum diluted to five μg/ml was immobilized on BioMag-anti human IgG-Fc beads and selected by a non-recombinant phage lysate (negative lysate). The enrichment monitored in this manner revealed an estimation of titer of the phages that would stick to a rich variety of IgG molecules provided by the serum. In other words, this type of control mimics the action of sticky clones, namely binding devoid of any affinity to the target, which in this case is the negative phage without fusion protein or peptide to display.

Enrichment factors in the range of two to ten fold, correspond to a minimum of 50 to 90 percent of all clones with a target specificity compared to the potential false positive. A minimum factor of two-fold enrichment was held as threshold considering the strategy applied and the level of stringency. It is suggested to sequence a minimum of 10 clones when ten-fold enrichment is observed (Dennis and Lowman, 2004). Accordingly 24 to 32 clones were analyzed in this study from the eluates of the last panning round.

4.5 Pilot CSF panning experiment

One further strategy applied in cDNA PD was to run a pilot selection to observe the nature of selection, maintained by the optimizations and adjusted level of stringency. The protocol of selection was tested using 4 CSF samples (2 unipolar depression, 2 NPH controls). As a secondary goal this pilot test would help

familiarization with sticky clones and clarify the baseline for the background coming from non-recombinant phages, and the phages that have affinity to matrix. Although prior to panning, the library was pre-cleared from bead reactive clones, there were some sticky clones resisting in both disease samples and the negative control. 9 clones were obtained from screenings of depressive patients, with no common clone in both patients. Both depression and NPH clones had 2 clones in common that were classified as CSF background which are independent of the disease (clones are listed in Chapter 5; Results).

4.6 Further fine-tuning: Compromising the number of selection rounds helps sustain weakly expressed peptides from being overrun by false positives

Despite all efforts to monitor and clean the sticky clones, there were always several false positives within the selected clones. This is partially due to two major factors: Initially, some clones might simply be underrepresented in the original library and high abundant competitors may outnumber some specific binders. Secondly, the phages have distinct capabilities to get propagated in the host when they are modified with different capsid fusion proteins. It is reasonable to assume that some clones might be more easily expressed while others might be toxic or deleterious to proper functioning of the phage. In summary, the phages with selective advantages but no specificity against the target may even out with real binders, and they would be carried away through the selection process.

Considering the uncontrollable selective bias favoring such false positives over true hits, the most crucial criteria after the quality of the original library is the number of selection rounds of panning. Passing beyond the optimal number of rounds (over-selection) may yield a smaller number of true hits at the expense of

many clones getting lost, introducing false positives that have escaped the natural selective pressure of binding affinity and were more successful in propagating. Such contaminating clones actually have no affinity to the target, and they may display totally unexpected properties (Russel M et al., 2004).

Strategically, three rounds of selection were used. The pilot experiments performed with CSF verified a reasonable enrichment after panning rounds three to four. To end the selection after three rounds would yield a heterogeneous mixture of clones; namely, true hits mixed with some background, and sticky clones that survived the stringent selection filter as false positives. Nevertheless, the aim of this modification was to observe the tendency of the selection pattern at the window of selection after round three, so that the true hits would not be lost even if they had a selective disadvantage compared to others.

After listing all the clones, a rigorous selection criteria was set by choosing the clones that repeatedly existed in the patient group and never in the control group list. Another criterion was finding different clones with overlapping or non-overlapping epitopes of the same hit, which was the case for a small number of hits.

4.7 Conclusion:

The efficiency of the cDNA PD using T7 select system and the overall feasibility of this strategy in finding autoantigens from CSF were addressed by a series of experiments. Several literature-based examples and this work demonstrated that the T7 cDNA PD is efficient and practical in finding autoantigens from heterogeneous human material. A special focus on CSF as a human sample, with low IgG content and limited sample size. Such limitations demanded adaptations and modifications to increase the sensitivity of the standard selection protocol.

Criteria confirmed for autoantigen selection by the T7 system with a special focus on limitations of CSF are:

1- Quality of the T7 Select cDNA library from normal brain

2- Sensitivity of the T7 select system

3- Ability of the matrix to maintain CSF-IgG without covalent attachment

4- Modifications on stringency in a balance that preserves the yield

5- Characterization of the background and sticky clones

There are many intrinsic limitations of PD as a technique and CSF as a sample which are not completely manageable. These limitations considerably decrease the sensitivity of PD while fostering the risk of high false positive rates. To overcome some of these factors, several modifications were tested to improve protocol and selection criteria. A critical attempt was to fractionate CSF by the MARS immunoaffinity separation which comprises both pros and cons. On one hand, the advantage of utilizing this precious human material in multiple applications is evident but, on the other hand, there is no way to avoid partial loss of CSF content and integrity. The evaluation of quantitative and qualitative differences of MARS treated and non-treated samples was compulsory for understanding the feasibility of this approach. Normal pressure hydrocephalus

(NPH) CSF samples were used in testing MARS depletion. After Vivaspin concentration, buffer exchange and MARS spin cartridge application, the protein amount was re-evaluated by BCA assay. The results showed a cumulative protein loss (Vivaspin plus MARS spin cartridge) around 15-20% compared to non-depleted CSF aliquot. Although spin cartridge separation was successful, analytical grade preparation by MARS HPLC was dissatisfactory with plaque lift assay results showing altered avidity. The alterations observed do not need to be universal for all IgG molecules in a CSF sample. But, for screening unknown autoantigen targets, such an alteration would introduce artifacts during sample preparation and greatly risk significance of the results.

Eventually, taking all the risks into account, the overall decision was to use whole CSF (crude sample) for selection. Even so, the MARS separation trials helped recognize that the MARS spin cartridge is suitable when CSF is strictly limited and has to be shared within several projects, provided that the results will not be affected by this intervention.

In summary, major contributions of the novel strategy applied in this study are:

1- Targeting optimal usage of CSF in several proteomics platforms (MARS)

2- *In situ* infection applied to autoantigen selection

3- Novel filtering systems for differentiating false positives from true hits, by

   a- avoiding accumulation of false positives with an approach to undermine the loss of true hits by fixing the panning to three rounds of selection for all samples, and

   b- setting a threshold of confidence for differentiating false positives from true hits, in terms of enrichment factors that compare a real selection in CSF to a control selection in serum mimicking generation of false positives.

Chapter 5

Results

5.1 Introduction

In order to identify disease-specific autoantigen candidates for affective disorders and SZ, 5 BPD and 6 SZ CSF samples were analyzed, as well as 2 major depression samples that were used in the primary pilot tests. In total, 21 samples from the non-AFD disease group were used as controls composed of multiple sclerosis, epilepsy, normal pressure hydrocephalus and others such as headache, somatoform disorder, cervical rhizopathy and shoulder-arm pain.

PD selection was optimized and tested for detection down to nanomolar range of autoantigen in CSF. As the limiting factor was CSF, repetition of panning and validation experiments, were dependent on the CSF volume available. Strategically 3 rounds of panning were always performed, which yielded a "snap shot" identification of the best fitting clones. Each selected clone from a given disease group was controlled in terms of absence of disease controls and sticky clones. The repetitive clones were evaluated for disease-specificity or sharing of common pathways. The best candidate clone was studied further with quantitative PCR and standard ELISA using recombinant autoantigen.

5.2 Enrichment of binding clones specific to CSF IgG

5 μg IgG from AFD/SZ and disease control CSF samples were immobilized on BioMag magnetic beads with an affinity to the Fc region of the human IgG molecule. The brain library displayed on T7 phage with $1.7x10^6$ pfu at 100-copy number was incubated with beads coupled to CSF IgG. Washing and elution conditions were tuned to the stringency level that had been tested and optimized, as given in the chapter on optimization. The concept based on the first panning round favoring yield over specificity, gradually shifted to utmost specificity in the following rounds. Titer assay was conducted after every panning round to monitor both the success of specific selection and, at the same time, the number of viable phage particles.

The enrichment over empty beads is the background enrichment and the enrichment over non-displaying phage is the marker enrichment. In this study, both types of enrichment were controlled at two levels. Firstly, the phage library was incubated with the BioMag-anti human IgG to wash out the clones that bind non-specifically to the matrix. After this step there was no further need for the assessment of the background enrichment as it was eliminated by the nature of the selection. Secondly, a separate set of BioMag-anti human IgG beads was coupled to human total serum, followed by incubation with non-displaying (negative) phage lysate. By conducting a titer assay, this strategy helps monitor the number of negative phages that stick to serum IgG non-specifically, but with a degree of affinity that survives the stringency of washing. Besides, serum is a more complex source than CSF with a huge variability of IgG subtypes, thereby contributing further to the likelihood of interactions yielding false positive results. Due to the fact that a positive selection marker for autoantigen selection was not

feasible, measuring the titer of false positive binding made it possible to set a threshold of confidence.

A factor of two-fold enrichment with respect to false positive selection marker was used as threshold of confidence. It also helped determine the number of clones to be analyzed, on the basis of the assumption that approximately one third of the final clones selected are specific to the target. Enrichment factor of an example selection is given below (Table 5.1). Figure 5.1 shows the selection efficiencies based on threshold of two-fold difference between enrichment factors of true selections versus false positive marker selection.

Table 5.1 Evaluation of enrichment with respect to marker for false positives. Counting well-separated plaques on an *E.coli* lawn gives the titer of the phage eluate that is conducted after each round of selection. The enrichment factor is calculated by dividing the ratio of titers of the samples (first round to last round) to the ratio of titer of the marker for the false positives.

	Titer round1 (pfu/ml)	Titer round 2 (pfu/ml)	Titer round 3 (pfu/ml)	Ratio (pan#1/pan#3)	Enrichment factor (ratio disease sample /
EPI 014	8×10^6	9×10^7	3×10^8	37,5	6,3
SZ 76	5×10^6	8×10^7	2,3	46	7,7
BP 203	3×10^6	1×10^8	2×10^8	66,7	11,1
EPI 300	1×10^7	9×10^7	2×10^8	20	3,3
Marker	5×10^5	2×10^6	3×10^6	6	1,0

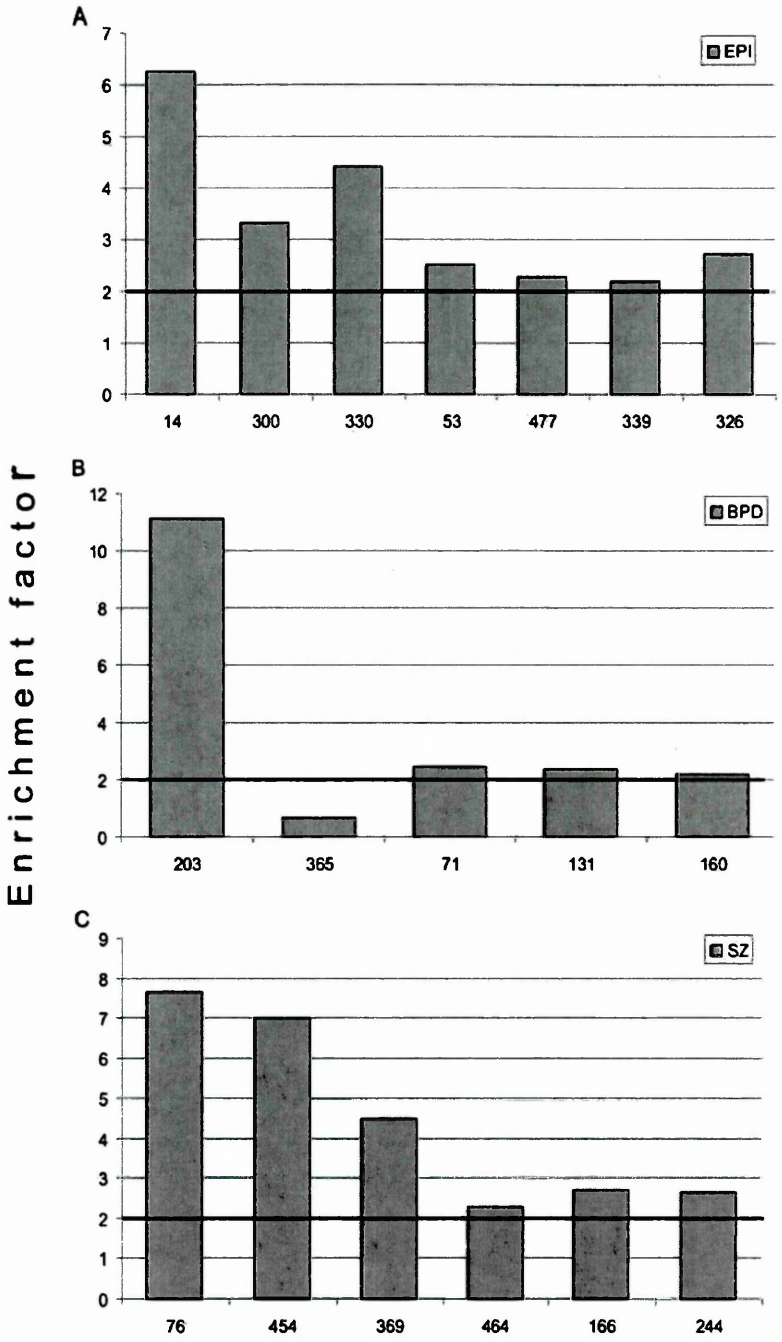


Figure 5.1 Enrichment of selection. A minimum 2-fold difference between the enrichment factors between disease and false positive marker was considered an indicator of true selection (bold line: threshold). X-axis shows the identity of CSF samples. A. Enrichment chart of epilepsy patients as a control group to AFD and SZ disease groups. B. BPD group showing relatively low enrichment efficiencies compared to EPI and SZ groups. CSF sample SZ #365 was considered more prone to include high numbers of non-specific interactions, as it was below the threshold. C. Enrichment factors associated with SZ disease group.

5.3 Identification of clones

After three rounds of panning, the plaques obtained by plating the eluate were picked for purification of DNA. Following a test PCR with primers flanking the multiple cloning site of T7Select10-3b vector, amplified insert was digested using MvaI restriction enzyme for fingerprinting. Both undigested clones, and the clones digested in different patterns, were selected for identification. Selected clones were amplified with PCR for sequencing, purified with the Qiaquick PCR purification system and sent to an external sequencing facility (Sequiserve GmbH). After the NCBI Blast search, the identified clones were listed according to disease groups and patients individually. Below is the listing of all clones obtained.

Table 5.2 All clones identified from major depression disorder (MDD) group.

Patient	Accession number	Protein ID
MD D 312	AAC51273	putative protein p150
	AAM22955	Rho-GTPase activating protein 10
	CAI41883	DEAH (Asp-Glu-Ala-His) box polypeptide 16
	AAQ91815	estrogen receptor 1
	A0MZ66	shootin-1
	NP_004732	nucleolar and coiled-body phosphoprotein 1, p130
	AB040934	KIAA1501 protein
	BC022348	KIAA1598 protein
MDD Lö	AAR95501	cytochrome c oxidase subunit I
	AAH07647	ADP-ribosylation factor 3
	AAI13669	proteasome activator subunit 4
	EAW68085	hydroxysteroid (17-beta) dehydrogenase 12
	NP_004732	nucleolar and coiled-body phosphoprotein 1 , p130
	AAA60350	proteolipid protein, myelin proteolipid protein (PLP or lipophilin)

Table 5.3 All clones identified from normal pressure hydrocephalus (NPH) control group.

Patient	Accession number	Protein ID
NPH 2	NP_000691	annexin A1
	NP_112420	heterogeneous nuclear ribonucleoproteinA1isoform b
	AAC51273	putative protein p150
	NP_004759	splicing factor, arginine/serine-rich 11
	CAE55174	v-myb myeloblastosis viral oncogene homologue
	BAC87650	unnamed protein product
NPH 3	AAH07501	protein-L-isoaspartate (D-aspartate) O-ethyltransferase , PCMT1
	AAM71140	neuregulin 1 isoform GGF2
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	XP_531360	hypothetical protein XP_531360 [Pan troglodytes]
	BAC85305	unnamed protein product

Table 5.4 All clones identified from epilepsy (EPI) control group.

Patient	Accession number	Protein ID
EPI 14/00	ABQ09374	haplotype H7 mitochondrion NADH dehydrogenase subunit 1
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_003624	ectodermal-neural cortex (with BTB-like domain)(ENC1)
EPI 053/05	NP_536341	septin 4 (SEPT4), transcript variant 3/hucep-7 mRNA for cerebral protein-7/cell division control-related protein 2b (hcdcrel2b)
	AAH93730	podocalyxin (PODXL)
EPI 330/04	NP_001001433	syntaxin 16 (STX16), transcript variant 1
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
EPI 477/01	NP_073568	nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1)
	NP_061054	calcium/calmodulin-dependent protein kinase II inhibitor 1 (CAMK2N1)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_006279	Thy-1 cell surface antigen (THY1)
	NP_001486	GDNF family receptor alpha 2 (GFRA2)

EPI 326/02	AAI30448	protocadherin alpha 10
	NP_057371	heterochromatin protein 1, binding protein 3 (HP1BP3)
	NP_003783	endothelial differentiation-related factor 1 (EDF1)
	XP_001134430	protein immuno-reactive with anti-PTH polyclonal antibodies
	AAH15491	small EDRK-rich factor 2 (SERF2)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_002859	RAB5B, member RAS oncogene family
	NP_703157	heparan sulfate 6-O-sulfotransferase 3 (HS6ST3)
EPI 339/04	NP_570824	synaptosomal-associated protein, 25kDa (SNAP25)
	NP_036269	dimethylarginine dimethylaminohydrolase 1 (DDAH1)
	NP_055180	heat shock 22kDa protein 8 (HSPB8)
	NP_647539	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (YWHAB)
	NP_036347	meningioma expressed antigen 5 (hyaluronidase) (MGEA5)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	AAP72185	migration-inducing protein 3
	AAH15491	small EDRK-rich factor 2 (SERF2)
	NP_114399	microtubule-associated protein 1B (MAP1B)
	ABQ09374	haplotype H7 mitochondrion NADH dehydrogenase subunit 1
	NP_477512	olfactomedin 2 (OLFM2)
	AAL37898	brain-specific GTP-binding protein
	AAH73834	CGI-96 protein
	NP_056049	G protein-coupled receptor 116

Table 5.5 All clones identified from schizophrenia (SZ) group.

Patient	Accession number	Protein ID
SZ 076/97	AAF27330	mitochondrial RNA-processing endoribonuclease RNA (RMRP) talin (TLN)
	AAH36243	interleukin 17D (IL17D)
	NP_001029197	endoplasmic reticulum protein 29 (ERP29)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_536341	septin 4 (SEPT4), transcript variant 3/hucep-7 mRNA for cerebral protein-7/cell division control-related protein 2b (hcdcrel2b)
	NP_003635	growth arrest-specific 7 (GAS7)
	NP_001070958	GNAS complex locus (GNAS)
	NP_207837	contactin associated protein-like 4 (CNTNAP4)
	AAI06032	ribosomal protein L17
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_071746	EF-hand calcium binding protein 1 (EFCBP1)
	BAD92272	myosin head domain containing 1 variant protein.
	AAI32828	coiled-coil domain containing 66
	NP_001393	eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)
	AAH17445	erythrocyte membrane protein band 4.9 (dematin)
	AAH15491	small EDRK-rich factor 2, (SERF2)
	AAH01811	RRS1 ribosome biogenesis regulator homolog
	BAD96295	TATA binding protein interacting protein 49 kDa variant/erythrocyte cytosolic protein of 54 kDa
	AAM28436	Tho2
	AAN01237	selectin E (endothelial adhesion molecule 1) (SELE)
SZ 454/00	AAF27330	mitochondrial RNA-processing endoribonuclease RNA(RMRP) talin (TLN)
	NP_736606	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10 (PSMD10)
	NP_001032411	ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase) (USP14)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	AAV97814	B-cell translocation gene 1, anti-proliferative (BTG1)
	AAH15491	small EDRK-rich factor 2 (SERF2)
	CAI41507	interleukin 3 receptor, alpha (low affinity) (IL3RA)

Patient	Accession number	Protein ID
SZ 369/98	NP_001958	eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2)
	NP_003077	MAP7 domain containing 2/small nuclear RNA activating complex, polypeptide 4, 190kDa (SNAPC4)
	AAH08749	myelin basic protein (MBP)
	NP_001020272	myelin basic protein (MBP)
	NP_002036	growth associated protein 43 (GAP43)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	AAH04357	zinc finger protein 410
	NP_001607	activin A receptor, type IIA (ACVR2A)
	AAH15491	small EDRK-rich factor 2 (SERF2)
	AAF59903	Intersectin 2
	NP_079508	receptor accessory protein 4 (REEP4)
	AAC25978	ES/130-related protein
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_003635	growth arrest-specific 7 (GAS7)
	NP_003169	synapsin II (SYN2)
	AAH32398	ATPase, H+ transporting, lysosomal V0 subunit a1
SZ 464/02	NP_061054	calcium/calmodulin-dependent protein kinase II inhibitor 1 (CAMK2N1)
	NP_004619	xeroderma pigmentosum, complementation group C (XPC)
	NP_004310	astrotactin 1 (ASTN1)
	AAI28578 AAC25978	ribosome binding protein 1 homolog 180kDa/ES/130-related protein
	AAH15491	small EDRK-rich factor 2 (SERF2)
	AAO13489	peroxisome proliferative activated receptor, alpha (PPARA)
	AAD30425	SH3 domain-binding protein SNP70
	NP_005648	tumor protein p53 binding protein, 1 (TP53BP1)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_207837	contactin associated protein-like 4 (CNTNAP4)

Patient	Accession number	Protein ID
SZ 166/02	AAI06032	ribosomal protein L17
	NP_057017	coiled-coil domain containing 72 (CCDC72),
	AAH15491	small EDRK-rich factor 2 (SERF2)
	AAB69001	T-cell receptor alpha/delta
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_207837	contactin associated protein-like 4 (CNTNAP4)
	NP_036419	centaurin, beta 2 (CENTB2)
SZ 244/04	NP_002678	pinin, desmosome associated protein (PNN)
	AAH15491	small EDRK-rich factor 2
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	AAH65700	Shwachman-Bodian-Diamond syndrome
	NP_055969	mesoderm development candidate 2 (MESDC2)

Table 5.6 All clones identified from bipolar disorder (BPD) group.

Patient	Accession number	Protein ID
BPD 203/05	NP_006470	RAD51 associated protein 1 (RAD51AP1)
	NP_690608	regucalcin (senescence marker protein-30) (RGN)
	AAB69001	T-cell receptor alpha/delta
	NP_002678	pinin, desmosome associated protein (PNN)
	AAH16660	heat shock 70kDa protein 8 (HSPA8)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_207837	contactin associated protein-like 4 (CNTNAP4)
	AAH01315	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)
	NP_000365	uroporphyrinogen decarboxylase (UROD)
BPD 071/02	AAH15491	small EDRK-rich factor 2 (SERF2)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	ABQ09374	haplotype H7 mitochondrion NADH dehydrogenase subunit 1
	AAH15177	ubiquinol-cytochrome c reductase hinge protein
	NP_477512	olfactomedin 2 (OLFM2)

Patient	Accession number	Protein ID
BPD 160/98	NP_116274	ATG4 autophagy related 4 homologue D (ATG4D)
	NP_004136	myosin IXB (MYO9B)
	AAH80654	myelin basic protein
	AAH21994	cytochrome c, somatic
	NP_002679 AAA20398	septin 5 (SEPT5) / glycoprotein Ib beta / cell division control related protein (hCDCrel-1)
	ABQ45201	haplotype W1 mitochondrion
	AAH15491	small EDRK-rich factor 2 (SERF2)
	NP_001633	amyloid beta (A4) precursor-like protein 2 (APLP2)
	NP_00102517 6 AAH12889	complexin 3 (CPLX3) / amyloid beta (A4) precursor-like protein 1
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	AAH10352 ABA60896	tyrosine 3-monooxygenase/tryptophan 5-monooxygenaseactivation protein beta polypeptide
	CAA32502	manganese superoxide dismutase (from SOD-2 gene)
BPD 365/04	NP_057371	heterochromatin protein 1, binding protein 3 (HP1BP3)
	AAW65373	matrix metalloproteinase 11 (stromelysin 3) (MMP11)
	AAH75862	calpain 1, (mu/I) large subunit
	NP_009151	carbonic anhydrase VB, mitochondrial (CA5B), nuclear gene encoding mitochondrial protein
	CAI41507	interleukin 3 receptor, alpha (low affinity) (IL3RA)
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	NP_003635	growth arrest-specific 7 (GAS7), transcript variant a
BPD 131/05	BAE78598	HLA-A gene for major histocompatibility complex, class I, A
	AAH07647	ADP-ribosylation factor 3
	NP_004168	syntaxin 3 (STX3)
	NP_002678	pinin, desmosome associated protein (PNN)
	AAT38112	RAD51-like 3 (RAD51L3)
	AAH15491	small EDRK-rich factor 2
	AAH01883	nucleolar and coiled-body phosphoprotein 1 NOLC1
	ABC40742	NADPH oxidase 1 (NOX1)
	NP_940852 AAB69018	apolipoprotein O-like (APOOL) / T-cell receptor alpha/delta
	AAF25871 AAC97946 AAB52589	lens epithelium-derived growth factor p52 / transcriptional coactivator p75 / autoantigen DFS70

Table 5.7 All clones identified from multiple sclerosis (MS) group.

Patient	Accession number	Protein ID
MS1	NP_009028	clathrin, light chain (CLTB)
	NP_003635	growth arrest-specific 7 (GAS7)
	AAH80654	myelin basic protein (MBP)
	AAH70049	LanC lantibiotic synthetase component C-like 2
	BAD92428	kinesin heavy chain isoform 5C variant
	AAH15491	small EDRK-rich factor 2 (SERF2)
	ABQ09374	haplotype H7 mitochondrion-NADH dehydrogenase subunit
	AAH15491	small EDRK-rich factor 2 (SERF2)
	AAI26226	SCC-112 protein
	AAH45759	MAP1LC3B protein
MS2	NP_0010701	neural cell adhesion molecule 1 (NCAM1)
	NP_207837	contactin associated protein-like 4 (CNTNAP4)
	NP_003635	growth arrest-specific 7 (GAS7)
	AAR85357	RNA-binding protein NOB1
	ABQ09374	haplotype H7 mitochondrion-NADH dehydrogenase subunit
	NP_733752	MAX-like protein X (MLX), transcript variant 3
	NP_004519	microsomal glutathione S-transferase 3 (MGST3)
	AAH18823	splicing factor, arginine/serine-rich 5
	AAD48774	3'-5' exonuclease TREX1
MS3	NP_963293	NDRG family member 2 (NDRG2)
	NP_872620	G patch domain containing 4 (GPATCH4)
	NP_004732	nucleolar and coiled-body phosphoprotein 1 (NOLC1)
	NP_073568	nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1)
	NP_477512	olfactomedin 2 (OLFM2)
	NP_057341	hippocalcin like 4 (HPCAL4)
	AAI08307	ring finger protein 130
	BAA82978	KIAA1026 protein

Patient	Accession number	Protein ID
MS4	NP_004990	myosin VI (MYO6)
	NP_036611	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma
	AAP69527	RRS1 ribosome biogenesis regulator homolog/pyruvate kinase RBC (PKLR)
	NP_055180	heat shock 22kDa protein 8 (HSPB8) alpha-crystallin-type heat shock proteins
	AAH15491	small EDRK-rich factor 2
	BAD92869	PI-3-kinase-related kinase SMG-1 isoform 1 homolog protein
	AAF36133	HSPC213
	NP_002678	pinin, desmosome associated protein (PNN)
	BAD92869	PI-3-kinase-related kinase SMG-1 isoform 1 homolog protein
	NP_071746	EF-hand calcium binding protein 1 (EFCBP1)
MS5	NP_000054	complement component 2 (C2)
	NP_064619	DTW domain containing 1 (DTWD1
	CAA49533	mitochondrial ATP synthase c subunit (P2 form)
	NP_006708	spindlin 1 (SPIN1)
	NP_002678	pinin, desmosome associated protein (PNN)
	NP_114381	eukaryotic translation initiation factor 4H (EIF4H)
	NP_057672	neuritin 1 (NRN1)
	CAA77750	human elongation factor 2
	AAA84914	pigment epithelium-derived factor
MS6	AAH47646	zinc finger protein 85 (ZNF85)
	NP_002374	MYC-associated zinc finger protein transcription factor (MAZ)
	AAI06081	high-mobility group nucleosome binding domain 1
	ABF14463	neighbour of BRCA1 gene 2
	NP_002678	pinin, desmosome associated protein (PNN)
	NP_000977	ribosomal protein L24 (RPL24)
	AAF43103	vanilloid receptor gene, partial sequence; CARKL and CTNS genes
	AAI12200	V-erb-a erythroblastic leukemia viral oncogene homologue 4 (avian) (ERBB4)

Patient	Accession number	Protein ID
MS7	AAH05916	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1
	AAH14512	stromal membrane-associated protein 1-like
	AAH15491	small EDRK-rich factor 2 (SERF2)
	AAH01883	nucleolar and coiled-body phosphoprotein 1
	EAW63602	neurofilament 3 (150kDa medium)
	NP_477512	olfactomedin 2 (OLFM2)
	NP_0010118	BTB (POZ) domain containing 1 (BTBD1)
	NP_056335	membrane associated guanylate kinase, WW and PDZ domain containing 1 (MAGI1)
	NP_002086	general transcription factor IIE, polypeptide 2, beta 34kDa (GTF2E2)
	NP_060914	protein phosphatase 2C, magnesium-dependent, catalytic subunit (PPM2C), nuclear gene encoding
	AAI39926	thymosin, beta 4, X-linked
	NP_002061	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2 (GNAI2)
MS8	NP_0010117	GTP-binding protein 9 (putative) (GTPBP9)
	NP_003635	growth arrest-specific 7 (GAS7)
	NP_000875	IMP (inosine monophosphate) dehydrogenase 2 (IMPDH2)
	NP_073568	nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1)
	NP_004719	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21 (DDX21)

Table 5.8 All clones identified from non-infectious disease control group.

Patient	Accession number	Protein ID
Headache (OND)	NP_000025	aldolase A, fructose-bisphosphate (ALDOA)
	CAC42117	NOD2 gene for LRR-containing protein, exons 1-11.
	NP_004719	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21 (DDX21),
	BAC06146	chromosome 19 clone CTD-3064H18/seven transmembrane helix receptor
	NP_065790	cell cycle progression 1 (CCPG1)
	NP_207837	contactin associated protein-like 4 (CNTNAP4)
	AAH15491	small EDRK-rich factor 2 (SERF2)
	NP_065778	zinc finger protein 295 (ZNF295)
	BAD92869	PI-3-kinase-related kinase SMG-1 isoform 1 homologue protein
	AAA84914	pigment epithelium-derived factor
	NP_003608	regulator of G-protein signaling 5 (RGS5)
	AAH08850	ribosomal protein L7
somatoform disorder (OND)	AAT45456	interleukin 12 receptor, beta 2 (IL12RB2)
	AAH00373	amyloid beta (A4) precursor-like protein 2
	ABQ09374	haplotype H7 mitochondrion-NADH dehydrogenase subunit
	NP_002678	pinin, desmosome associated protein (PNN)
	AAH15491	small EDRK-rich factor 2
	NP_892116	eukaryotic translation initiation factor 5 (EIF5) transcript variant2
	AAH99907	general transcription factor II
	NP_071746	EF-hand calcium binding protein 1 (EFCBP1)

Patient	Accession number	Protein ID
cervical rhizopathy (OND)	NP_057589	zinc finger, CCHC domain containing 17 (ZCCHC17)/HSPC243
	NP_002678	pinin, desmosome associated protein (PNN)
	NP_064541	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1 (C1GALT1)
	NP_612370	leucine rich repeat and sterile alpha motif containing1 (LRSAM1)
	NP_715624	potassium voltage-gated channel, Shaw-related subfamily, member 2 (KCNC2)
	NP_006097	Yes-associated protein 1, 65kDa (YAP1)
	NP_004719	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21(DDX21)
	AAC50968	KIT protein and alternatively spliced KIT protein (KIT)
	AAH95420	splicing factor, arginine/serine-rich 5
	NP_003606	solute carrier family 4, sodium bicarbonate cotransporter, member 7 (SLC4A7)
	ABI78944	SEC16L (SEC16L)
	NP_056982	zinc finger and BTB domain containing 7A (ZBTB7A)
	ABQ09374	haplotype H7 mitochondrion-NADH dehydrogenase subunit
	ABK41936	CD40 ligand (TNF superfamily, member 5, hyper-IBM syndrome) (CD40LG)
	ABQ45202	haplotype W1 mitochondrion NADH dehydrogenase subunit 2
shoulder-arm pain (OND)	ABK41936	CD40 ligand (TNF superfamily, member 5, hyper-IBM syndrome) (CD40LG)
	ABQ45202	haplotype W1 mitochondrion NADH dehydrogenase subunit 2
	NP_001767	ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1)
	AAH15491	small EDRK-rich factor 2 (SERF2)

5.4 Selected clones

5.4.1 Repetitive clones

The overall distribution of identified clones among different disease groups primarily yielded a group of clones that were repetitively selected with hit frequency ≥ 2.

Secondly, there were clones from a family of proteins that were exclusively selected from disease groups, although each clone was not repetitive when taken alone. For instance, clones the products of which belong to heat shock, ribosomal, myosin, cytochrome c and zinc finger family proteins compromise a large portion of all selected clones.

Non-frequent hits were of two classes: One class involved clones that were detected with different transcriptional variants, either overlapping or non-overlapping clones encoding the same protein. The other class involved few clones that qualify as multiple hits only when they are considered collectively with a one-time hit matching only one of the control samples. These clones were listed as repetitive clones due to the fact that the control disease samples were as heterogeneous as disease samples and it could not be totally excluded that a control subject might have a subtle autoimmune response to a candidate autoantigen, albeit showing no pathological phenotype. Table 5.9 represents all repetitive clones falling into explained categories.

Table 5.9 Repetitive clones. The list of all repetitive clones identified after three rounds of panning with hit frequency ≥2 among different disease groups. Sticky clones are given to indicate the uniform presence of such background interactions throughout the experiment.

Identification		Disease Groups						
		SZ n=6	BP n=5	MS n=8	EPI n=7	MDD n=2	NPH n=2	Other n=4
Talin (TLN)		2	-	-	-	-	-	-
Septin 4/Septin5 (SEPT4/SEPT5)		1	1	-	1	-	-	-
Growth arrest-specific 7 (GAS7)		2	1	3	-	-	-	-
T-cell receptor alpha delta locus		1	2	-	-	-	-	-
Pinin, desmosome associated protein (PNN)		1	2	4	-	-	-	2
Heat shock	70kDa protein8 (HSPA8)	-	1	-	-	-	-	-
	22kDa protein 8 (HSPB8)	-	-	1	1	-	-	-
Contactin associated protein-like 4 (CASPR4/CASPR3)		3	1	1	-	-	-	1
Interleukin	17D (IL17D)	1	-	-	-	-	-	-
	3 receptor, alpha (low affinity)	1	1	-	-	-	-	-
Heterochromatin protein 1, binding protein 3 (HP1BP3)		-	1	-	1	-	-	-
Olfactomedin 2 (OLFM2)		-	1	2	1	-	-	-
Nuclear casein kinase and cyclin-dependent kinase substrate1 (NUCKS1)		-	-	2	1	-	-	-
Calcium/calmodulin-dependent protein kinase II inhibitor 1		1	-	-	1	-	-	-
Myelin basic protein (MBP)		1	1	1	-	-	-	-
Ribosomal protein	L17	2	-	-	-	-	-	-
	L24	-	-	1	-	-	-	-
Ribosome binding protein 1 homologue 180kDa/ES/130-		2	-	-	-	-	-	-
EF-hand calcium binding protein 1 (EFCBP1)		1	-	1	-	-	-	1
Myosin	IXB (MYO9B)	1	-	-	-	-	-	-
	VI (MYO6)	-	-	1	-	-	-	-
Coiled-coil domain containing	66 (CCDC66)	1	-	-	1	-	-	-
	72 (CCDC72)							

Identification		Disease Groups						
		SZ n=6	BP n=5	MS n=8	EPI n=7	MDD n=2	NPH n=2	Other n=4
Protease (prosome, macropain) 26S subunit, non-ATPase, 10 (PSMD10)		1	-	-	-	-	-	-
cytochrome c	reductase hinge protein (ubiquinol)	-	1	-	-	-	-	-
	somatic	1	-	-	-	-	-	-
Haplotype, mitochondrion NADH dehydrogenase	W1, subunit2	1	-	-	-	-	-	1
	H7, subunit1	-	1	2	2	-	-	2
Splicing factor, arginine/serine-rich 5		-	-	1	-	-	-	1
PI-3-kinase-related kinase SMG-1 isoform 1homolog protein		-	-	1	-	-	-	1
Zinc finger protein	410 (ZNF410)	1	-	-	-	-	-	-
	85(ZNF85)	-	-	1	-	-	-	-
Amyloid beta (A4) precursor-like	protein 1 (APLP1)	-	2	-	-	-	-	1
	protein 2 (APLP2)							
General transcription factor II		-	-	1	-	-	-	1
Tyrosine3 monooxygenase /tryptophan 5-monooxygenase activation protein, beta		-	-	1	1	-	-	-
Lens-,(p52) or transrciptional coactivator p75	epithelium-derived growth factor	1	-	-	-	-	-	-
Pigment-		-	-	1	-	-	-	1
Nucleolar and coiled-body phosphoprotein 1 (NOLC1)		33						
Small EDRK-rich factor 2		25						

5.4.2 Major sticky clones

A number of sticky clones are uniformly present in the eluates from almost all samples from any type of disease or control group. Table 5.10 shows the most common sticky clones.

Table 5.10 Major sticky clones of T7 library. Nucleolar and coiled-body phosphoprotein p130 is the most prominent background clone as it was encountered in almost all samples, and in optimization experiments carried out using negative lysate (empty T7 phages).

Non specific clones (BG)	SZ	BP	MS	EPI	MDD	NPH	Other
Nucleolar and coiled-body phosphoprotein, NOLC1, p130	+	+		+	+	+	+
Haplotype (H7) mitochondrion NADH dehydrogenase subunit 2	+	+	+	+			+
Small EDRK-rich factor 2	+	+	+	+			+

5.4.3 Pseudo genes, novel genes and uncharacterized proteins

The PD selection survey in Blast yielded many clones that are translated into unknown proteins with novel or pseudo gene identity. These clones were not studied in further detail except categorizations according to genomic locations (Table 5.11) and repeated hit frequency within disease groups (Table 5.12).

Table 5.11 Distribution of selected pseudo/novel/unidentified genes. Below is the number of unknown genes selected by PD listed according to their genomic locations.

Chromosome	SZ (n=6)	BP (n=5)	MS (n=8)	EPI (n=7)	MDD (n=2)	NPH (n=2)	Other (n=4)
1	1	-	9	1	2	-	4
2	4	2	3	2	-	-	6
3	2	3	2	1	-	1	1
4	2	1	2	1	1	-	3
5	3	-	1	4	-	-	2
6	-	2	2	2	1	-	4
7	7	1	4	1	-	-	2
8	3	1	2	3	-	-	1
9	3	1	2	3	-	-	2
10	1	-	5	-	-	-	1
11	3	2	2	1	-	-	1
12	3	-	4	4	-	-	1
13	2	-	3	1	-	-	1
14	-	-	5	1	-	-	-
15	-	-	1	-	-	-	-
16	1	1	1	2	-	-	2
17	2	-	4	-	1	-	-
18	-	-	1	-	-	-	-
19	1	3	2	-	1	-	2
20	-	-	-	2	-	-	1
21	-	-	-	-	-	-	-
X	1	-	1	-	1	-	4
Y	-	-	-	-	-	-	1

Table 5.12 Distribution of repeatedly selected pseudo/novel/unidentified genes among disease groups. Upper left part shows AFD, SZ or MS specific clones with no hit in control samples. Bottom left part shows other repetitive clones that are not SZ/BPD specific.

Clone	Chromosome	SZ	BP	MS	MDD	EPI	NPH	Other
BAC RP11-241J12	**5**	2						
BAC clone RP11-708P17	**7**	1		1				
PAC clone RP4-754G14	**7**			2				
clone RP11-131N11	**10**	1		1				
BAC RP4-601P9	**12**	1		1				
BACR-158A24 of library RPCI-11	**14**			2				
clone CTC-471J1	**19**		2					
BAC clone RP11-762E8	**2**			1				1
BAC RP11-321A2	**3**		1					1
clone RP3-422G23	**6q24**			1		1		
PAC clone RP5-1102B4	**7**	1						1
clone RP11-134N2	8	1				1		
clone RP11-354P17	**9**	2		2		1		
genomic DNA, RP11-822I2	**11q**	1				1		1
BAC RP11-641A6	**12**	1				1		
clone RP11-533D19	16							2
clone RP1-274L7	**X**			1				2

5.4.4 Clonality

Plaque selection performed after PD assay from each patient yielded a number of clones. Plaques were picked randomly and clones were subsequently sorted according to the size of the insert and the restriction pattern after PCR amplification. Only undigested clones and a representative clone from each restriction analysis were sequenced. Clones were found to represent known genes as well as unknown gene products and pseudo genes. Table 5.13 shows the quantitative representation of clonality that represents the frequency of the same hit per patient. In the table, the number of clones picked, sequenced and identified known gene products are listed. This is followed by the frequency of highly enriched clones for each patient, which is obtained from the analysis of undigested or same size PCR products. Regarding the frequency of the identical restriction digestion patterns, MBP was found as the clone with the highest enrichment compared to other selected hits. Not included are the background and sticky clones, unknown gene products and pseudo genes.

Table 5.13 Clonality in terms of quantitative evaluation of identical clones picked from each patient

Samples	Clones			APLP	Cytochrome C	HSP70	XPC	SH3 binding protein SNP70	ERP29	RPL17	Talin	MBP	Caspr4	Gas7	TCRA/D	Il3RA
	Total number picked	Total number sequenced	Identified	Number of identical sized PCR products								Number of identical restriction patterns				
SZ076	48	32	20						2	2			1	1		
SZ454	24	9	7								2					1
SZ369	48	30	16					2				3		1		
SZ464	24	14	10				2						1			
SZ166	24	17	7										1		1	
SZ244	24	13	5													
BPD203	24	14	9			2							1		1	
BPD365	30	14	7											1		1
BPD071	30	15	5													
BPD160	24	16	12	2	2							1				
BPD131	24	18	10												1	

Abbreviations: APLP: amyloid beta protein precursor, HS70: heat shock protein 70, XPC: Xeroderma pigmentosum complementation group C, ERP29: endoplasmic reticulum protein 29, RPL17: ribosomal protein L17, MBP: myelin basic protein, Caspr4: contactin associated protein4, GAS7: growth arrest specific 7, TCRA/D: T cell receptor alpha/delta, IL3RA: interleukin 3 receptor alpha.

5.4.5 Gene ontology profiling

AFD, BPD, MDD and MS related repetitive clones were classified. The results yielded a rather broad distribution of clones. Therefore, a GO classification was performed to see the overall distribution of selected clones. As shown in Figure 5.2, the prominent groups were found to be structural, adhesion (17%), followed by immune specific and ATP-related (13%) proteins.

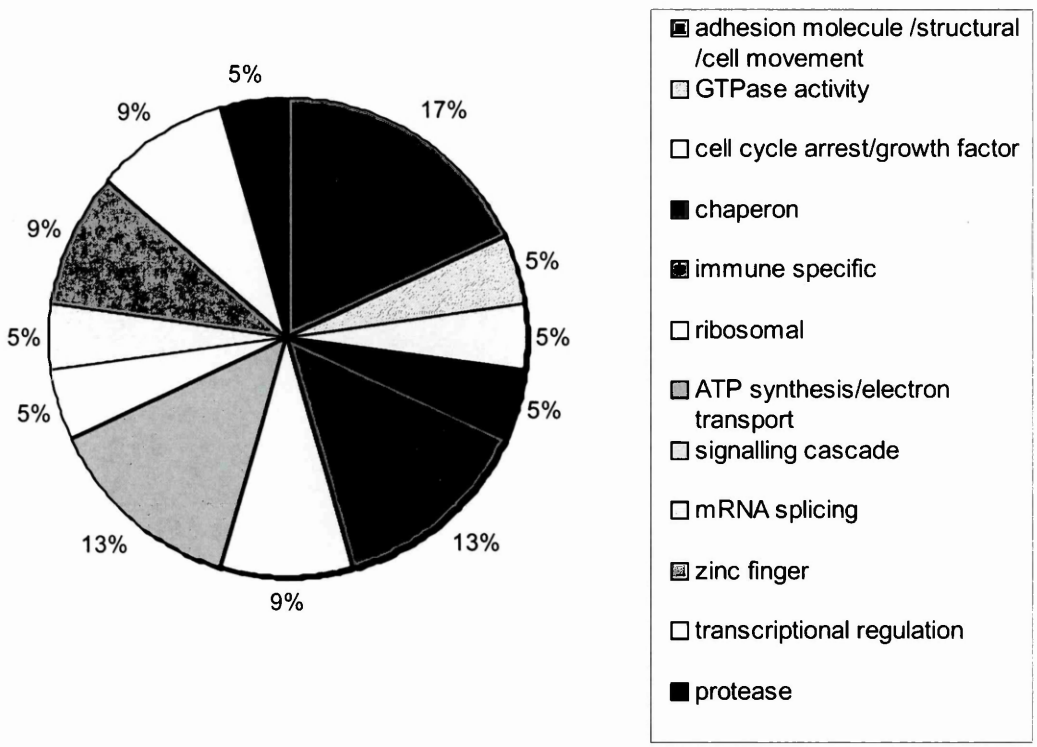


Figure 5.2 GO distributions of repetitive clones. All of the clones depicted within this scheme are autoantigen candidates that are categorized only according to their GO, irrespective of their hit frequency.

5.5 BPD and SZ related autoantigen candidate profiles

There were 7 molecules that were classified as frequent hits among 11 samples (5 BPD, 6 SZ). Table 5.14 demonstrates that these six clones have the potential to act as candidates with high hit frequencies.

Table 5.14 Overview: The selected autoantigen profiles that have a hit frequency ≥2 selected from AFD and SZ, with co-reactivity to MS samples.

	TLN	IL3RA	GAS7	CASPR4/ CASPR3	TCRA/D	MBP	RPL17
SZ 454							
SZ 076							
SZ 464							
SZ 166							
SZ 244							
SZ 369							
BP 203							
BP 365							
BP 071							
BP 160							
BP 131							
MS1							
MS2							
MS3							
MS4							
MS5							
MS6							
MS7							
MS8							

5.5.1 Clones with hit frequency 4 within the BPD/SZ disease group

Contactin associated protein or cell recognition protein 4 (CNTNAP4 or CASPR4) isoform1 (NP_207837) was repeatedly selected from 4 patient samples, as 3 hits from 6 SZ patients and 1 hit from 5 BPD patients. Caspr3 was also identified by Blast by 85% homology to the sequence obtained from PD selection. Caspr4 and Caspr3 are, therefore, both considered as hits, although Caspr4 is more often referred to for simplicity. Caspr4/Caspr3 displaying phage was selected from one MS patient's CSF and headache control.

```
Caspr4          DDCDDPLVSALPQASFSSSSELSSSHGPGFARLNRRDGAGGWSPLVSNKYQWLQIDLGER 60
Caspr3          -DCDSPLASALPRSSFSSSSELSSSHGPGFSRLNRRDGAGGWTPLVSNKYQWLQIDLGER 59
                 ***.**.****::****************:***********:*****************

Caspr4          MEVTAVATQGGYGSSNWVTSYLLMFSDSGWNWKQYRQEDSIWGFSGNANADSVVYYRLQP 120
Caspr3          IEVTAVATQGGYGSSDWVTSYLLMFSDGGRNWKQYRREESIWGFPGNTNADSVVHYRLQP 119
                :**************:***********.* ******:*:*****.**:******:*****

Caspr4          SIKARFLRFIPLEWNPKGRIGMRIE 145
Caspr3          PFEARFLRFLPLAWNPRGRIGMRIE 144
                .::******:** ***:********
```

Figure 5.3 Caspr4 and Caspr3 amino acid sequences obtained form PD selection. The 145 amino acids long autoantigenic target sequence maps to N-terminal aa30 to aa173. Among this region, Caspr4 and Caspr3 have sequence identity with score 85 as determined by CLUSTAL 2.0.5 Multiple Sequence Alignment. "*" are the identical residues or nucleotides in that column, ":" are conserved substitutions, and "." are semi-conserved substitutions.

5.5.2 Clones with hit frequency 3 within the BPD/SZ disease group

Growth arrest specific protein 7 (Gas7) (NP_003635), and T-cell receptor alpha/delta (TCRA/D) (AAB69001, AAB69001, AAB69018) were selected repeatedly from 3 SZ/BPD patients. Gas7 was found among MS clones 3 more times, while TCRA/D was restricted to the SZ/BPD disease group only.

5.5.3 Clones with hit frequency 2 within the BPD/SZ disease group

Myelin basic protein (MBP) was selected from 1 BPD and 1 SZ patient's CSF (AAH08749 and NP_001020272: SZ patient, AAH80654: BPD patient). Additionally, 1 MS patient was responding to MBP (AAH80654).

Talin (AAF27330) was selected from 1 BPD and 1 SZ patient. The CSF of the same SZ patient reacted with Interleukin 3 receptor alpha (IL3RA) (CAI41507) and, together with another SZ patient, IL3RA was detected exclusively within the SZ group with a hit frequency of 2.

Ribosomal protein RPL17 was identified from 2 SZ patients. According to PD selection, one of the patients (SZ 076) that reacted with RPL17 was also reactive with Caspr4, Talin and Gas7. Similarly the other patient (SZ 166) was reactive with Caspr4 and TCRA/D as well.

5.6 Validation of selected clones by phage ELISA

After categorization of clones the most prominent candidate autoantigen was assessed for confirmation with alternative immunological methods.

After a PD selection, phage ELISA is the classical option for such a query. Accordingly, the primal goal was to try to reproduce findings from the PD selection using phage ELISA on Caspr4/Caspr3 displaying phage particles. Consequently, the CSF from 4 patients yielding Caspr4/Caspr3 as an autoantigen candidate was tested. Caspr4/Caspr3 displaying phage lysate was purified by CsCl gradient and incubated with anti-T7 tail coated strip well ELISA plates. As controls, Gap43 displaying phage and negative lysate (empty phage) were used. Caspr4/Caspr3 positive crude CSF samples from 4 positive patients were individually used as detection antibody source. CSF was pre-incubated with empty T7 phage before application.

The reaction was amplified by a biotin-streptavidin step, using anti-human IgG-biotin and streptavidin-HRPO complex, and detected by TMB at λ=450 nm.

In agreement with PD selection, Caspr4/Caspr3 positive CSF showed a more pronounced reaction with Caspr4/Caspr3 displaying phage compared to others ($p<0.05$, Student t-test) (Figure 5.4). On the other hand, high background noise was observed that came from negative control lysate Gap43, empty phages and blank (wells coated with anti-T7tail antibody), that compromised the signal.

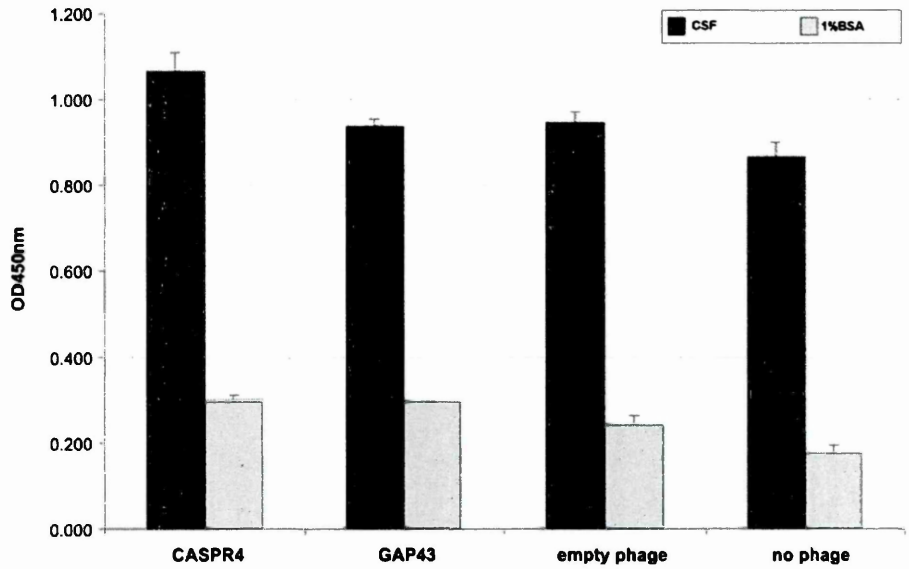


Figure.5.4 Phage ELISA testing CSF samples that yielded Caspr4/Caspr3 as autoantibody candidates according to the PD selection. Small scale Caspr4/Caspr3, Gap43 and empty phage lysates were purified by CsCl gradient and 1:20 dilution was applied on ELISA wells pre-coated with IgG2a T7 tail fiber monoclonal antibody. CSF of Caspr4/Caspr3 positive patients, pre-cleared of empty T7 phage (negative lysate), was used as detection antibody source. A cocktail of 1:1000 diluted polyclonal (Jackson laboratories) and 1:500 diluted monoclonal (BD Biosciences) antihuman-IgG-biotin was applied for 2 hrs and subsequently incubated with streptavidin-HRPO (BD Biosciences) at 1:2000 dilutions for 25 min.

5.7 Validation by conventional ELISA using recombinant Caspr4 peptide

A conventional ELISA, that tests the reactivity of CSF from PD positive patients against Caspr4 recombinant peptide, was applied. For this purpose, the sequence within the N-terminal epitope of caspr4 that corresponds to 61 amino acids from the discoidin domain was cloned. After bacterial expression and purification by His-Tag absorption, the recombinant peptide was concentrated and buffer exchanged to PBS. After confirmation by western blot using anti-his antibody, the peptide was coated in 1%PBS-BSA to Corning High-Bind microtiter plates.

One CSF sample that was positive for rCaspr4 peptide according to the PD was used in comparison to a negative CSF from the epilepsy group. Regarding the IgG content, two concentrations were used: 1 μg/ml and 10 μg/ml. 2 μg/ml rCaspr4 was used as target. Similar to phage ELISA, signal to noise enhancement by biotin coupled anti-human IgG and streptavidin-HRPO incubation was preferred (Figure 5.5).

Next, 7 μg/ml rCaspr4 was used as target to analyze PD positive SZ and BPD samples and a comparison at fixed IgG concentration (12 μg/ml IgG) of CSF from disease and control disease groups was performed (Figure 5.6).

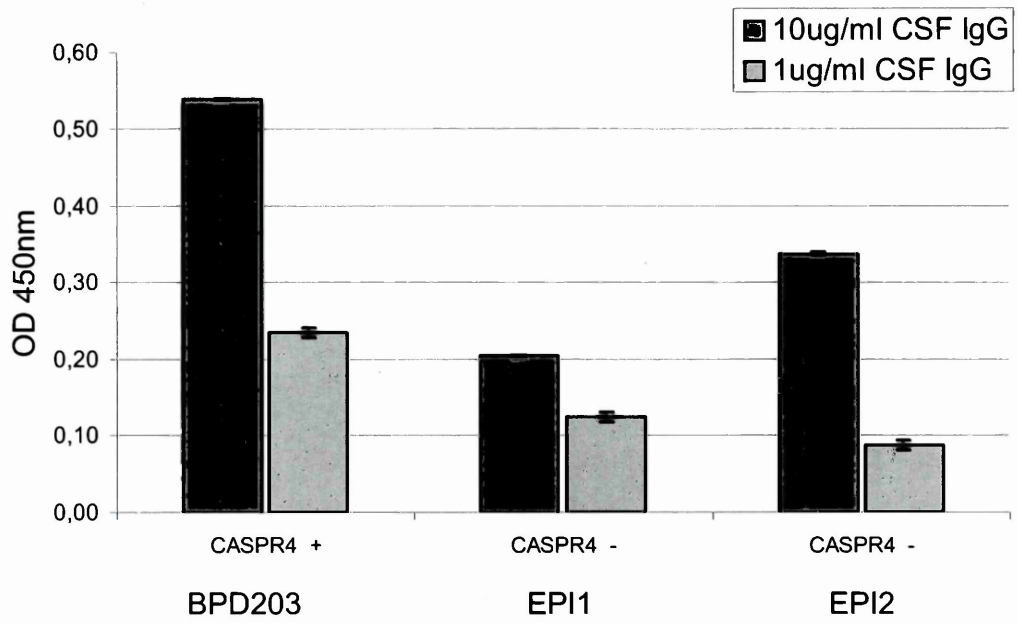


Figure 5.5 2μg/ml recombinant Caspr4 peptide mapping N-terminal peptide from aa1 to 60, was coated on Corning High-Bind 96-well microtiter plates. CSF samples were diluted in blocking buffer at concentrations of 10 μg/ml and 1 μg/ml IgG. Caspr4+ CSF designates BPD patient 203, identified with PD selection, as positive for Caspr4 autoantigen. Caspr4+ CSF containing 1μg/ml IgG dilution reacted stronger with Caspr4 peptide than the EPI patient 1, with 10 μg/ml IgG dilution, implying that up to ten-fold reactivity is observable. The experiment was performed in duplicate, results representing the average values.

The results indicated that the Caspr4 recombinant peptide may act as an immunogenic epitope for Caspr4 reactive CSF selected by PD. Different CSF samples, either positive or negative for Caspr4, had a different reactivity for the rCaspr4 peptide. However, a trend for higher reactivity of SZ/BPD CSF than the control disease groups existed (t-test, p=0.14).

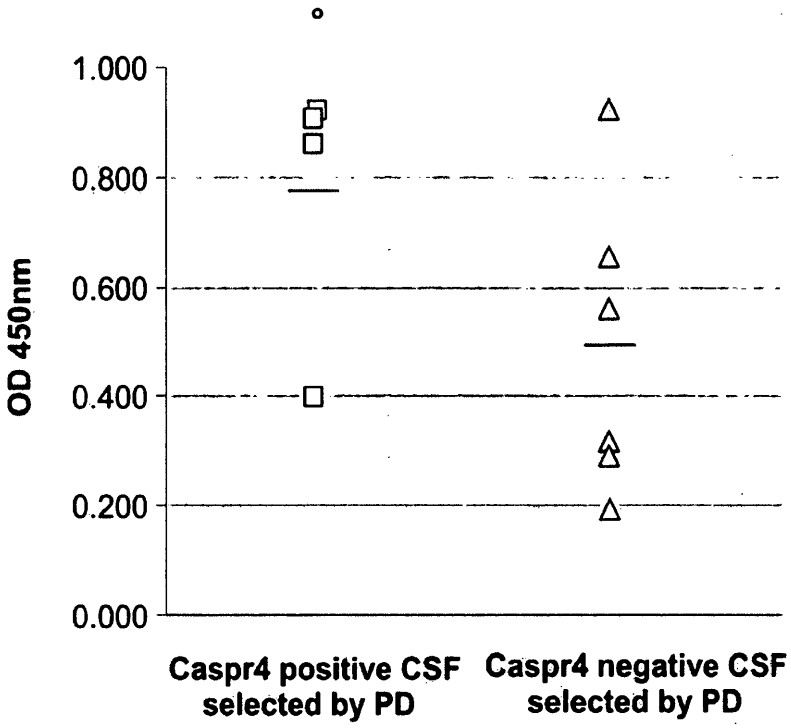


Figure 5.6 CSF reactivity to recombinant Caspr4 peptide. 7μg/ml rCaspr4 peptide was coated on 96-well microtiter plates. After blocking, CSF samples were added (IgG concentration adjusted to 12μg/ml). Anti-human IgG-Biotin (Jackson laboratories) used to detect CSF IgG bind to rCaspr4 peptide. The results showed that, there is a trend for SZ/BPD group to yield a higher signal intensity compared to the disease control group, indicating an autoantigenic response (p=0.14, two tailed test).

5.8 Differential autoantigenic characteristics of N-terminus of Caspr family proteins

After validation of the reaction to the rCaspr4 peptide, we questioned whether other members of the Caspr family have the same autoantigenic property. For this purpose, two commercially available recombinant peptides, Caspr1 and caspr2 were used. The whole amino acid sequence of caspr1 and caspr2 has 38 and 37 % homology to caspr4 and caspr3, respectively. rCaspr1 peptide maps to 20-70 aa at the N-terminus of Caspr1 of human origin. rCaspr2 has 50 aa size and maps to the immediate N-terminus of human Caspr2.

Caspr4 recombinant peptide has a size of 61 aa and it was cloned from human genomic DNA. The aa sequence homology of the N-terminal amino acids of recombinant Caspr4, Caspr and Caspr2 are given in Table 5.15.

Table 5.15 Homologies of Caspr family members at N-terminus compared by ClustalW2 multiple sequence alignment.

Seq A	Name	aa sequence	Length (aa)	Seq B	Name	Length (aa)	Score
1	Caspr1	WGYYGCDEELVG PLYARSLGASSYY SLLTAPRFARLHGI SGWSPRIGDPNP	51	2	Caspr2	51	23
2	Caspr2	SCLCRAWTAPSTS QKCDEPLVSGLPH VAFSSSSSISGSYS PGYAKINKRGG	51	3	Caspr4	61	37
3	Caspr4	MGSVTGAVLKTLL LLSTQNWNRVEAG NSYDCDDPLVSAL PQASFSSSSELSS SHGPGFARL	61	1	Caspr1	51	29

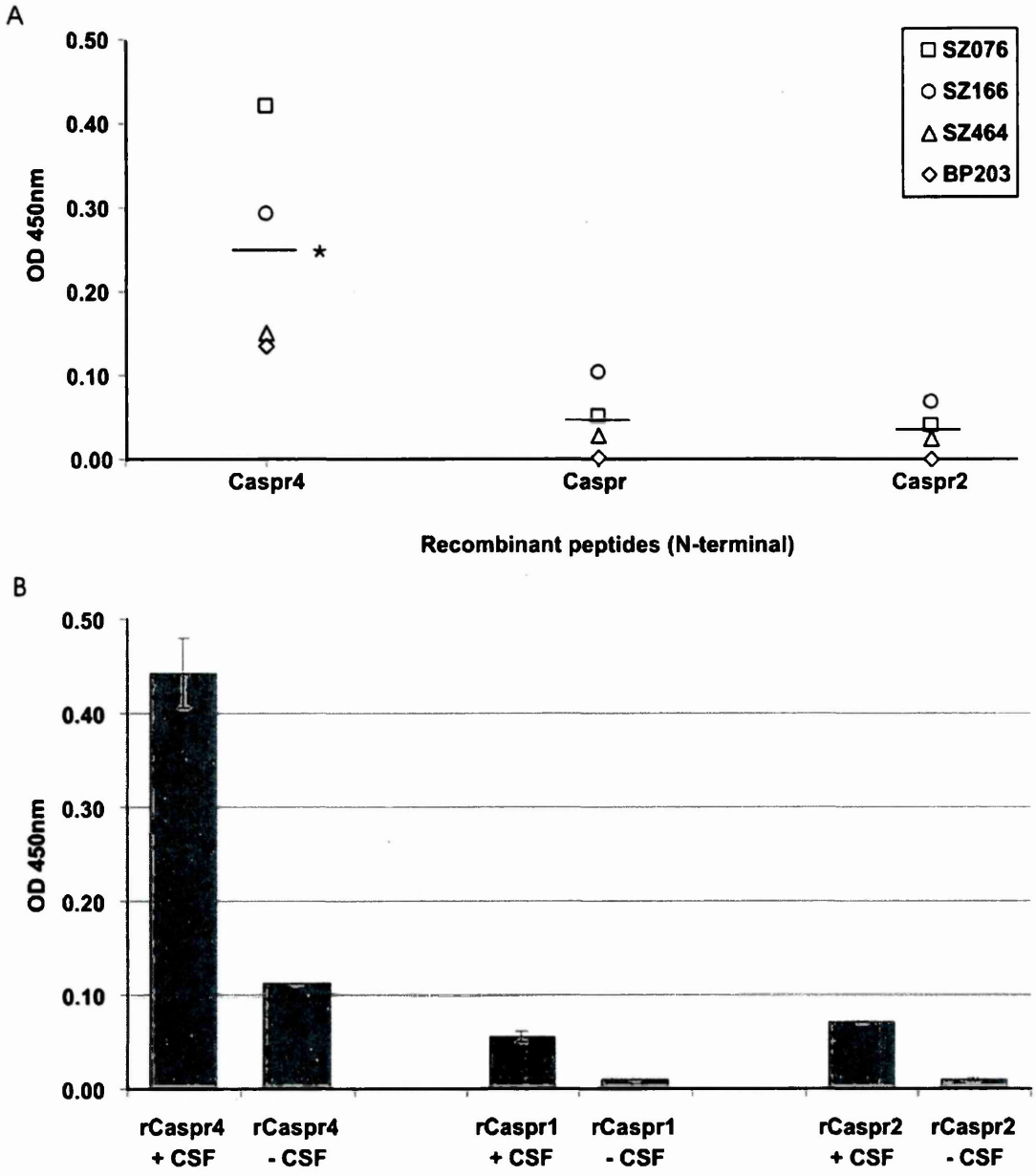


Figure 5.7 Recombinant Caspr4, Caspr and Caspr2 peptides at 1μg/ml concentrations were coated on Corning HighBind 96-well microtiter plates. A. CSF samples from SZ/BPD patients that were identified with PD selection as positive for Caspr4 autoantigen were tested. All the recombinant peptides represent N-terminal discoidin domains, conserved among Caspr family proteins There was a significant response to Caspr4 epitope compared to signal from homologous recombinant peptides ($p<0.05$, two-tailed t-test). The experiment was performed in duplicate and repeated twice, results representing average values and showing SEM B. CSF from BPD patient 203 showed more reactivity to rCaspr4 than rCaspr1and rCaspr2 and control wells with no CSF, indicating epitope selectivity.

As depicted in Figure 5.7, a significant difference in reactivity against the epitope obtained by PD selection in comparison to homologue sequences from close family members was observed ($p<0.05$, two tailed test). This implicates that the N-terminal sequence of Caspr4 obtained after PD selection, may act as a highly specific autoantigen target.

5.9 Quantitative PCR analysis of expression pattern of myelination related genes

In order to understand the expression pattern of relevant myelin-related genes, Caspr1, Caspr4, MBP and DDR1 in an integrated way, a quantitative PCR approach was applied. In this way it is possible to assess the differential expression of these genes from birth until early adulthood in mice. Three male and three female mice were analyzed at PND1, PND7, PND14, PND21, PND28, PND35 and PND42, thus allowing observation of the possible difference between sexes. The whole brain and spinal cord cryo-sections were used for RNA isolation. Expression of CASPR1, CASPR4, DDR1 and MBP was compared to GAPDH expression.

In the brain, CASPR1 and MBP mRNA levels showed a similar increased pattern of expression during the first two postnatal weeks (Figure 5.8). In the following weeks, CASPR1 levels stayed constant at peak levels while MBP expression dropped back to initial values. DRR1 and CASPR4 had a linear expression pattern after the first postnatal week. Females had a higher expression of both genes during the first postnatal week.

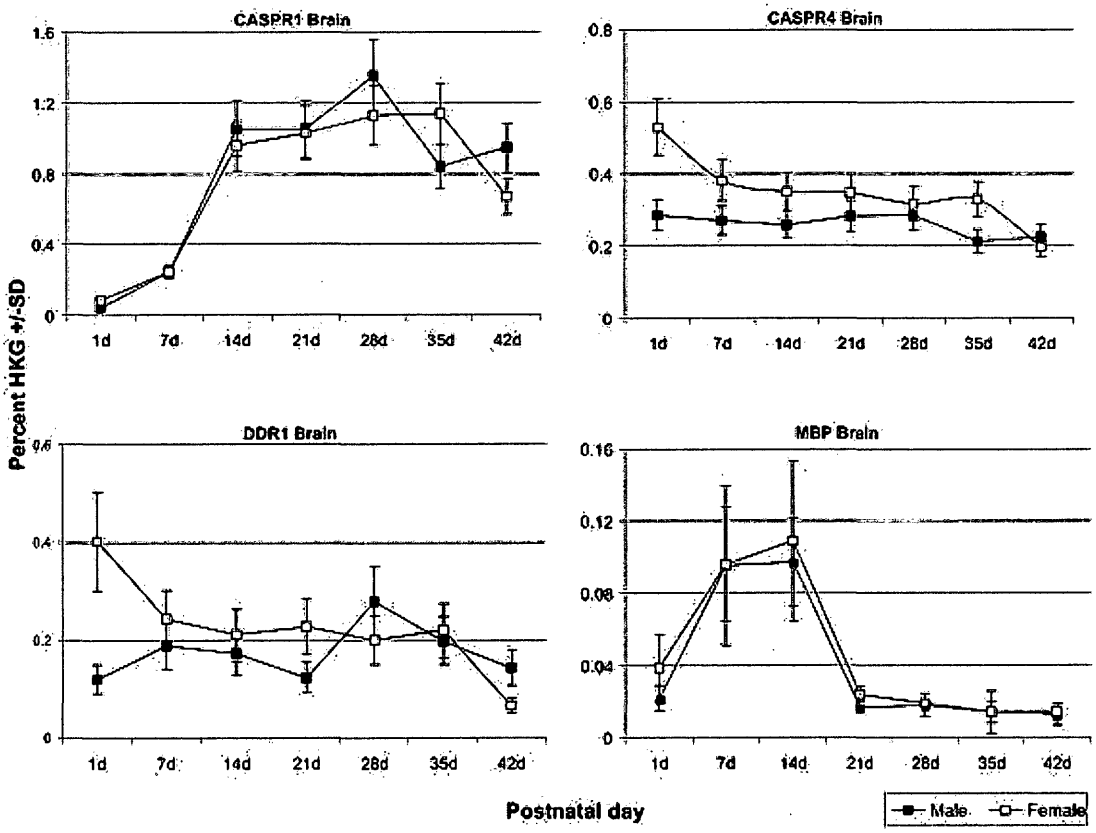


Figure 5.8 Caspr1 and MBP transcripts show a similar expression pattern from birth to postnatal day 14, afterwards Caspr1 levels stayed constant while MBP expression dropped back to initial values. DRR1 and Caspr4 show a linear expression pattern, females have a higher expression of both genes during first postnatal week. Values represent percent gene expression level relative to housekeeping gene (HKG), error bars show standard deviation. For each data point n=3.

In spinal cord all four genes showed a similar expression pattern from birth to PND42 in males and females. The only remarkable difference at the mRNA level was observed at PND7, where females had a higher CASPR1 and CASPR4 expression (Figure 5.9).

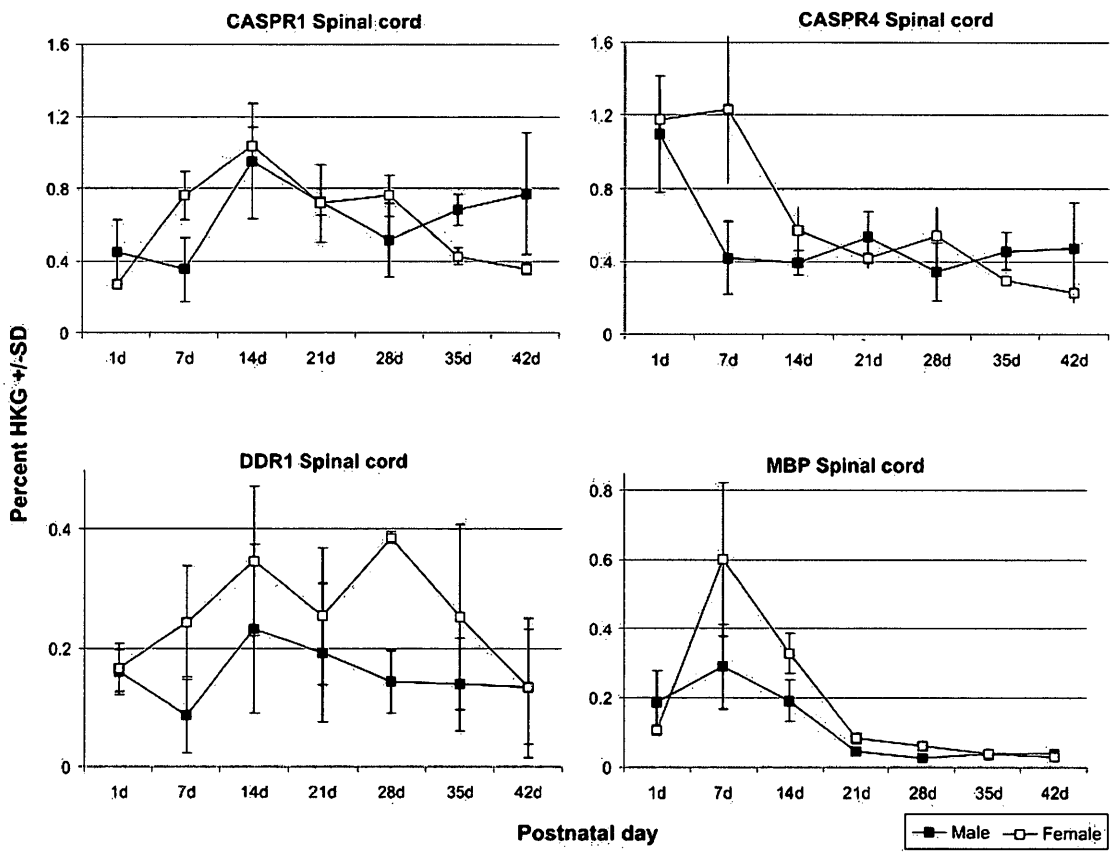


Figure 5.9 Caspr1, Caspr4, DDR1 and MBP transcripts show similar expression patterns from birth to PND42 for males and females. The only difference at the mRNA level is observed at PND7, where females have a higher Caspr1 and Caspr4 expression. Values represent percent gene expression relative to housekeeping gene (HKG), error bars show standard deviation. For each data point n=3.

When female and male mice were grouped together, Caspr1 expression in the brain was higher than three other genes and reached a peak value at PND28. In spinal cord Caspr4 expression was highest at PND1 and constantly dropped until PND14 when Caspr 1 reached a peak. Caspr4 and DDR1 expression levels and patterns were very similar in the brain. Caspr1 and MBP had different expression levels in the spinal cord but the patterns of expression were similar with MBP having highest expression at PND7 and Caspr1 at PND14, before they were down-regulated and stabilized at PND21 (Figure 5.10).

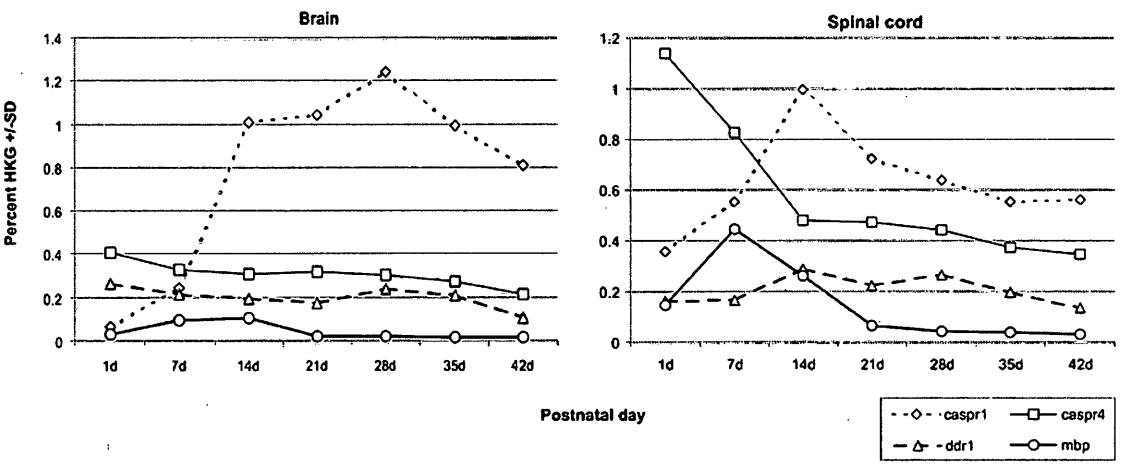


Figure 5.10 In this figure, results from male and female mice have been grouped together. In brain tissue, Caspr4, DDR1 and MBP show similar expression through out the postnatal developmental period. Caspr1 is strongly unregulated and reaches a higher level at PND14, continues until the end of week 4, and then starts to drop linearly (left). In spinal cord Casp1 and Caspr4 do the opposite as seen by a constant down-regulation of Caspr 4 until PND14 in contrast to up-regulated Caspr1. Values represent percent gene expression relative to housekeeping gene (HKG). For each data point n=6.

5.10 SUMMARY

1. Caspr4 and Caspr3 were identified as candidate autoantigens (Caspr3 as a homologue with an 85% identical amino acid sequence) by phage display selection.
2. Extracellular 145 amino acids at the N-terminus of Caspr4/Caspr3 were mapped as the autoantigen target in a subset of SZ/BPD patients.
3. Phage ELISA, using purified Caspr4/Caspr3 positive lysates, showed reactivity against phages that display Caspr4.
4. CSF from SZ/BPD patients that were selected by PD, reacted towards the rCaspr4 epitope with a trend that was higher than the control disease group ($p<0.15$).
5. Conventional ELISA, using recombinant Caspr4 peptide and its close homologues suggested a specific autoantigenic activity of the epitope obtained by PD selection. A significant response ($p<0.05$) by PD positive CSF samples towards rCaspr4, but not rCaspr1 and rCaspr2 peptides, was observed.
6. Caspr4 epitope resides within the discoidin (DISC) domain. Another SZ related protein with a discoidin domain is called DDR1 (also TrkE), which is encoded by a myelin gene. Myelin basic protein is also a candidate autoantigen selected by PD assay, albeit with a smaller hit frequency ($n=2$) than Caspr4.
7. Quantitative PCR analysis of Caspr4, MBP and DDR1 showed expression and gender differences, especially during the first two weeks of post-natal brain development in mice.

Chapter6

RZPD Protein Array: Method and Results

6.1 Introduction

RZPD Protein Array consists of approximately 28,000 proteins printed in duplicate on 22cm x 22cm PVDF membranes. The human fetal brain library consists of over 38016 clones spotted on two membranes each having a capacity of 27648 different clones or proteins.

RZPD protein arrays are used in several studies including target protein identification using antibodies and sera, identification using antibodies or antibodies purified from serum, functional assays, e.g. phosphorylation, ribosylation, methylation (Lee et al., 2002), identification of DNA/RNA binding proteins (Buessow et al., 2004) and protein-protein interaction studies (Grelle et al., 2005).

Before this study, others have applied RZPD protein array in screening of autoantibodies using human sera (Buessow et al., 2004), but not CSF. Similar to designated modifications constructed for the PD assay for this study, the protein array protocol had to be adopted for CSF conditions. Initially, 4 samples were tested but first attempts yielded no signals. Subsequent optimization trials were successful, so that 4 SZ, 3 BPD and 3 EP CSF samples were analyzed by RZPD at their facilities in Heidelberg.

6.2 RZPD Protein array flow chart

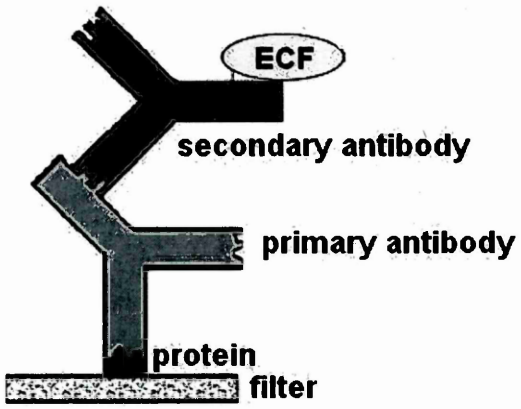


Figure 6.1 Principle of the array (source: RZPD).

6.2.1 Quality control of macroarray

1. Incubation of macroarray with anti-His antibody
2. Incubation with anti-mouse antibody, linked to alkaline phosphatase
3. Incubation with ECF substrate
4. Scanning

6.2.2 Characterization of antibody (epitope mapping)

1. Incubation of macroarray with antibodies to be characterized (mouse monoclonal antibody, rabbit antiserum, rat antiserum)
2. Incubation with secondary antibody (anti-mouse, anti-rabbit, anti-rat), linked to alkaline phosphatase
3. Incubation with ECF substrate
4. Scanning

6.2.3 Serum screening for autoantibodies

Pre-absorption of human serum against *E.coli* proteins is recommended: Alternatively, immobilized E. coli lysate can be used (Pierce, No. 44938 or 44940).

1. Incubation of macroarray with human CSF (or plasma, serum, etc)
2. Incubation with anti-human antiserum, linked to alkaline phosphatase
3. Incubation with ECF substrate
4. Scanning

Note: Dilutions for CSF screen were adjusted individually.

6.3 CSF Samples

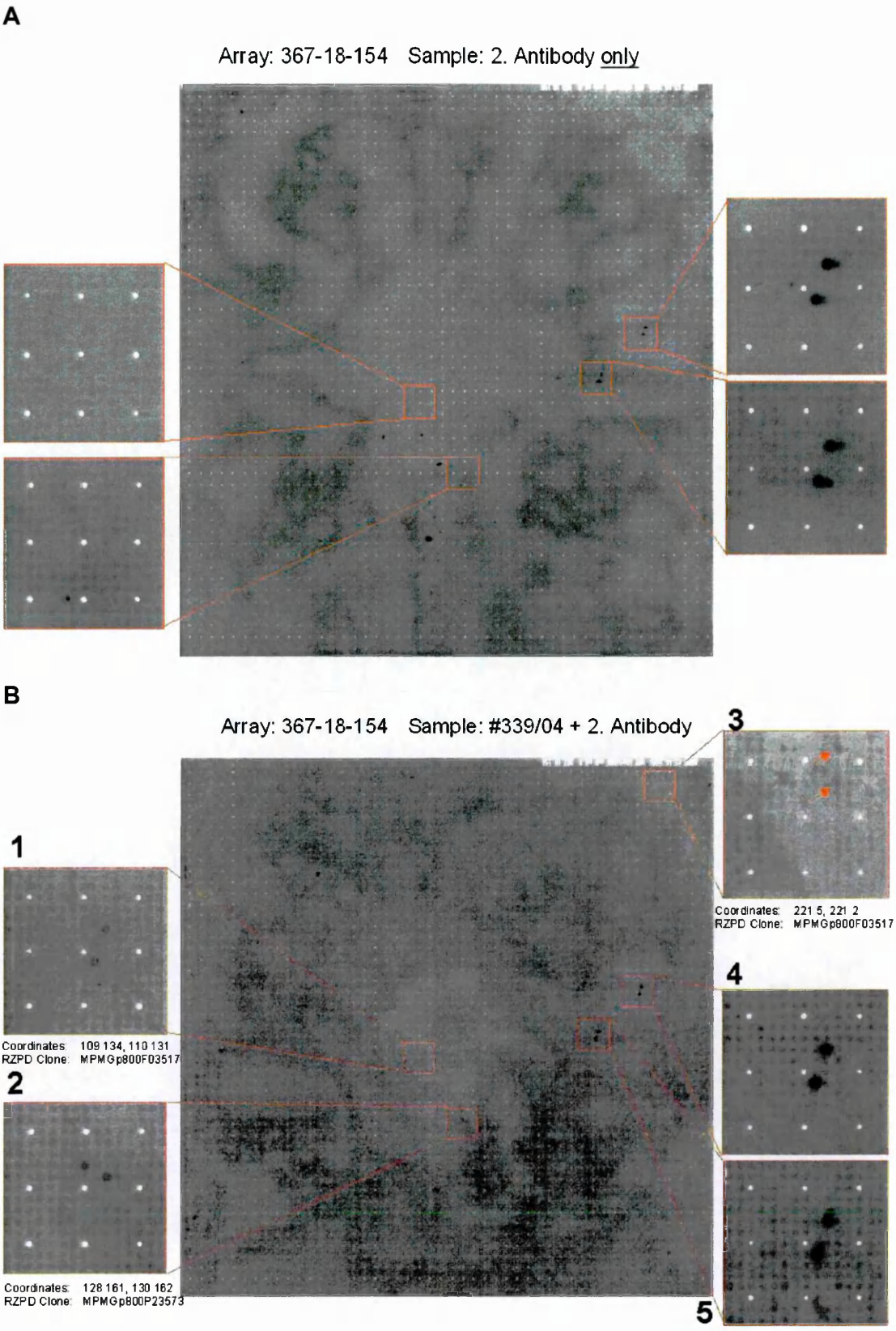
Table 6.1 CSF samples analyzed by RZPD protein array

Sample	IgG µg/ml	Albumin mg/l	Total protein mg/l	Gender	Age	Volume CSF analyzed (µl)
SZ 454/00	22.7	147	280	F	34	600
SZ 166/02	28.7	230	420	M	56	450
SZ 464/02	45.6	424	520	F	24	350
SZ 244/04	23.3	166	278	F	61	650
SZ 369/98	53,0	348	493	F	43	Test
SZ 076/97	24,6	265	587	M	50	Test
BP 365/04	20.3	188	298	F	48	750
BP131/05	18.9	137	199	F	64	800
BP203/05	11.9	161	223	F	46	1200
BP 071/02	25,4	231	320	F	52	Test
EP 014/00	26.6	225	304	F	25	600
EP 477/01	34	205	340	M	17	450
EP 330/04	31.9	289	411	M	46	500
EP 339/04	61,0	265	428	F	56	Test

Note that, during optimization trials and screening, the samples were analyzed anonymously; i.e., not knowing the disease groups they belonged to. Identified proteins and corresponding clones are listed as annotated clone lists with coordinates of spots on the filters. Protein array image and sequence analyses with subsequent Blast searches were carried out.

6.4 Results and Discussion

a. Filter images



C

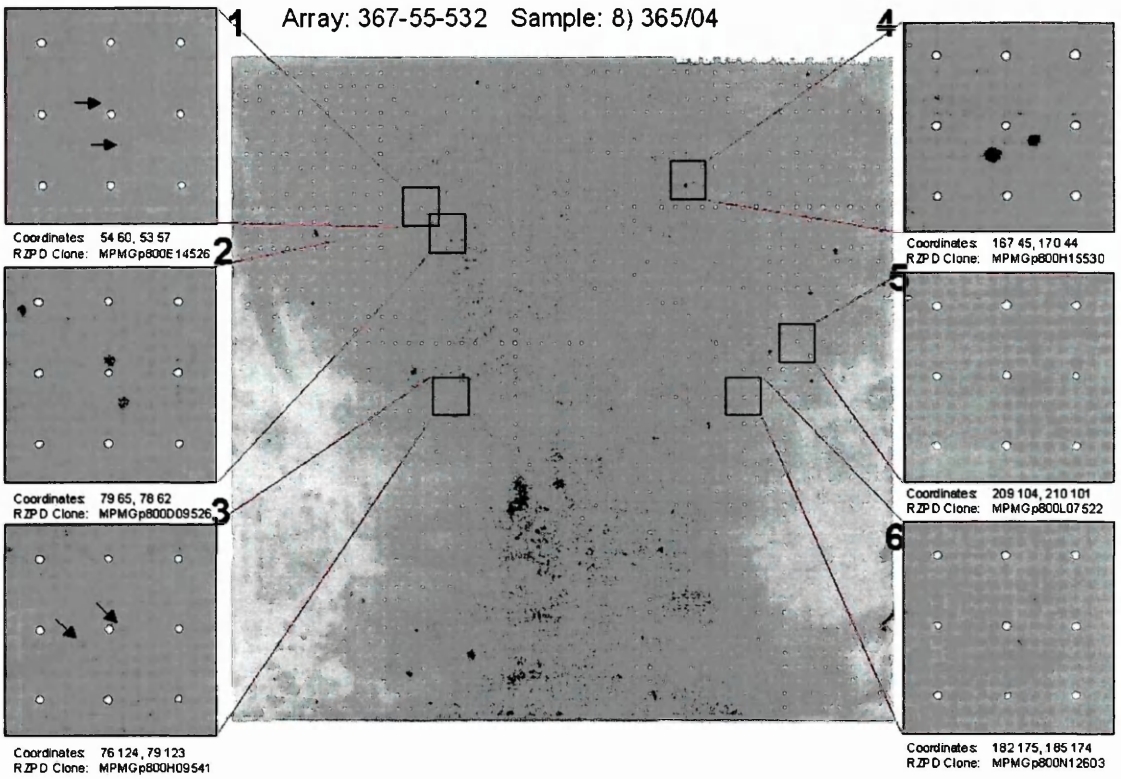


Figure 6.1 Protein array filter. Approximately 38,000 clones were distributed on two membranes, each with ~27,500 clones in duplicate spots (~55,000 spots in total). Each filter had an identity code **A.** Secondary antibody control of the 367-18-xyz filter series. The membrane was solely incubated with secondary antibody to detect coordinates of non-specific binding clones. The second filter did not show any reactivity to secondary Ab (data not shown). **B.** The same membrane after incubation with CSF showing reactive clones in duplicate. Coordinates of hit 1 and 3 are different. However, the 2 hits have the same clone identity (p800F03517), making this clone a strong positive hit. The second membrane yielded no positive hits (data not shown). Sample 339 is an epilepsy patient CSF sample showing autoimmune reactivity to MHC class I antigen (NCBI accession CAC17463). **C.** An example of a membrane displaying multiple responses to BPD CSF probe. The signal intensities were still moderate despite optimizations. All of the clones identified are listed in the overall list of identified RZPD clones.

b. Identified clones

Protein array results were evaluated manually and signals with significant intensities sequenced by RZPD. The signals were persistently weak. The criterion was the identification of the same protein twice on one membrane, making it more likely to be a real signal. Table 6.2 lists the Blast results of significant spots from all repetitive (hit frequency ≥2) samples.

Table 6.2: All repetitive clones resulting from the RZPD protein array screen of CSF samples.

Clone		BP (n=4)	SZ (n=6)	EPI (n=4)
caspase 6 isoform beta		3	2	2
Ufm1-conjugating enzyme 1	clone1	3	2	-
	clone2	4	3	3
ankyrin repeat and SAM domain-containing protein 6		1	3	1
OTU domain, ubiquitin aldehyde binding	clone 1	2	1	-
	clone 2	3	1	-
B-cell CLL/lymphoma11A iso3		2	-	1
proline-rich acidic protein 1		3	1	-
Stathmin-like 4	clone1	1	2	1
	clone2	-	2	1
	clone3	-	2	1
	clone4	-	2	1
similar to GluR- (N12603) delta2 philic-protein		2	4	1
myosin light polypeptide 6B		1	-	1
histone deacetylase 10		1	3	2
histone deacetylase		-	2	-
MYC-associated zinc finger protein	clone1	-	1	1
	clone2	-	1	1
	clone3	-	1	1
	clone4	-	3	1
5'-nucleotidase domain containing protein 2		-	2	-
LOC387790 protein		1	1	-
hCG2031974		-	1	1
ribosomal protein S12		-	2	1
ribosomal protein S27a		-	2	-
serine/threonine protein phosphatase 1 regul.subun.10		-	1	1
zinc finger protein 358		-	1	1
zinc finger protein 259		2	-	-

Clone	BP (n=4)	SZ (n=6)	EPI (n=4)
capicua protein	2	-	-
glyceraldehyde-3-phosphate dehydrogenase	3	-	-
RD RNA-binding protein, Negative elongation factor E	3	-	-
G1 to S phase transition 1, peptide chain release factor 3	2	-	-
CUE domain containing 2, HOYS6 and HOYS7	3	-	-
splicing factor, arginine/serine-rich 11 and glutamine-rich	1	1	-
sperm-associated antigen 7	2	-	-
Ras-related GTP binding A and B	2	-	-
PIP5K1C protein	2	-	-
eukaryotic translation elongation factor 1 gamma and 2	-	2	-
ProSAAS precursor (dynamin 1 and 2)	1	2	-
leucine rich repeat containing 47	-	2	-
superoxide dismutase copper chaperone	-	2	-
mitogen-activated protein kinase 11, p38B MAP kinase	-	2	-
eukaryotic translation initiation factor 3 and 4a-I	-	2	-
phospholipase C gamma 1 isoform a	3	1	-
neuron navigator	1	2	-
NME1-NME2 protein, non-metastatic cells 2, protein	-	2	-

c. Disease-specific repetitive clones:

The set of SZ/BPD specific clones, identified from at least two patients by RZPD protein array is shown in Table 6.3. The list below shows that several spots that belong to the same clone can be obtained from one membrane or can be distributed to two membranes.

Table 6.3: Disease-specific repetitive clones resulting from RZPD protein array screen of CSF samples.

Clone	RZPD clone ID
G1 to S phase transition 1, peptide chain release factor 3, (GSPT1)	MPMGp800H18563, MPMGp800M10544, MPMGp800P20554
RD RNA-binding protein, Negative elongation factor E (NELF-E)	MPMGp800F17571, MPMGp800A16590, MPMGp800D13558
splicing factor, arginine/serine-rich 11 and glutamine-rich nuclear protein (p54)	MPMGp800M23569, MPMGp800P18528

Clone	RZPD clone ID
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	MPMGp800B23574, MPMGp800P16540
CUE domain containing 2 (HOYS6, HOYS7)	MPMGp800I07557, MPMGp800J05545, MPMGp800H15591, MPMGp800A22590, MPMGp800P10578, MPMGp800O2356, MPMGp800I09541, MPMGp800E09558
Sperm-associated antigen 7 (SPAG7)	MPMGp800O02506, MPMGp800F05518, MPMGp800N12598
Ras-related GTP binding A and B (RRAGA)	MPMGp800J19525 MPMGp800K01582 MPMGp800A24596 MPMGp800J24581
PIP5K1C protein, TATA box binding protein (TBP)-associated factor	MPMGp800J12548, MPMGp800L23591
Eukaryotic translation elongation factor 1 gamma and 2 (EEF2)	MPMGp800O02544 MPMGp800C17586
NME1-NME2 protein, non-metastatic cells 2 protein (NM23B)	MPMGp800O05526, MPMGp800H16529
OTU domain ubiquitin aldehyde binding 1 (MBD3)	MPMGp800E13553, MPMGp800K13541
5'-nucleotidase domain containing 2 (NT5D2)	MPMGp800J09580, MPMGp800K16585
Capicua protein (CIC)	MPMGp800P10599, MPMGp800C18591
ProSAAS precursor (dynamin 1 and 2) (PCSK1)	MPMGp800P22510, MPMGp800H19595
leucine rich repeat containing 47 (LRC47)	MPMGp800H21569, MPMGp800F22533

Clone	RZPD clone ID
superoxide dismutase copper chaperone (CCS)	MPMGp800A03589, MPMGp800L02569
mitogen-activated protein kinase 11, p38B MAP kinase (MAPK11)	MPMGp800N14589, MPMGp800C06553
histone deacetylase (HDAC)	MPMGp800E12566
Eukaryotic translation initiation factor 4A (eIF4A-I), 3	MPMGp800D08553, MPMGp800I16577
neuron navigator (NAV1)	MPMGp800A11507

d. Common clones to phage display selection:

1. Eukaryotic translation initiation factor 4A isoform 1 and isoform 2:

Eukaryotic translation initiation factor 4A isoform 1 was selected from 2 SZ patients by protein array (PA), and isoform 2 was selected from 1 SZ patient by PD assay. Blast alignment of DNA sequences from PD and protein array yielded an 85% sequence homology. Multiple alignment by Clustal 2.0.5 revealed the common amino acid sequence of both initiation factors (figure 6.2).

```
PD              MSGGSADYNREHGGPEGMDPDGVIESNWNEIVDNFDDMNLKESLLRGIYAYGFEKPSAIQ 60
PA              MSASQDSRSRDNG-PDGMEPEGVIESNWNEIVDSFDDMNLSESLLRGIYAYGFEKPSAIQ 59
                **... . .*::* *:**:*:************.******.******************

PD              QRAIIPCIKGYDVIAQAQSGTGKTATFAISILQQLEIEFKETQALVLAPTRELAQQIQKV 120
PA              QRAILPCIKGYDVIAQAQSGTGKTATFAISILQQIELDLKATQALVLAPTRELAQQIQKV 119
                ****:******************************:*:::* ******************

PD              ILALGDYMGATCHACIGGTNVRNEMQKLQAEAPHIVVGTPGRVFDMLNRRYLSPKWIKMF 180
PA              VMALGDYMGASCHACIGGTNVRAEVQKLQMEAPHIIVGTPGRVFDMLNRRYLSPKYIKMF 179
                ::********:*********** *:**** *****:********************:****

PD              VLDEADEMLSRGFKDQIYEIFQKLNTSIQVVLLSATMPTDVLEVTKKFMRDPIRILVKKE 240
PA              VLDEADEMLSRGFKDQIYDIFQKLNSNTQVVLLSATMPSDVLEVTKKFMRDPIRILVKKE 239
                ******************:******:. **********:*********************

PD              ELTLEGIKQFYINVEREEWKLDTLCDLYETLTITQAVIFLNTRRKVDWLTEKMHARDFTV 300
PA              ELTLEGIRQFYINVEREEWKLDTLCDLYETLTITQAVIFINTRRKVDWLTEKMHARDFTV 299
                *******:********************************:********************

PD              SALHGDMDQKERDVIMREFRSGSSRVLITTDLLARGIDVQQVSLVINYDLPTNRENYIHR 360
PA              SAMHGDMDQKERDVIMREFRSGSSRVLITTDLLARGIDVQQVSLVINYDLPTNRENYIHR 359
                **:************************************************************

PD              IGRGGRFGRKGVAINFVTEEDKRILRDIETFYNTTVEEMPMNVADLI 407
PA              IGRGGRFGRKGVAINMVTEEDKRTLRDIETFYNTSIEEMPLNVADLI 406
                ***************:******* **********::****:******
```

Figure 6.2 Amino acid sequence similarity of eukaryotic translation initiation factor 4A isoform 2 (PD) and isoform 1 (PA) by ClustalW2 multiple sequence alignment with a score of 89. Both factors were exclusively selected from the SZ disease group and were absent from the epilepsy control group. "*" are the identical residues or nucleotides in that column, ":" are conserved substitutions, and "."are semi-conserved substitutions.

2. Eukaryotic translation elongation factor gamma and alpha:

Eukaryotic translation elongation factor gamma was selected by PD while the alpha isoform was selected by protein array. Both genes encode a subunit of the elongation factor-1 complex. Translation elongation factor gamma contains an N-terminal glutathione transferase domain which may be involved in regulating the assembly of multi-subunit complexes containing this elongation factor and aminoacyl-tRNA synthetases. Eukaryotic translation elongation factor alpha has two isoforms. Isoform alpha 1 is expressed in brain, placenta, lung, liver, kidney and pancreas, and alpha 2 is expressed in brain, heart and skeletal muscle. Isoform 1 was identified as an autoantigen in 66% of patients with Felty syndrome that is defined by rheumatoid arthritis with an enlarged spleen and abnormally low white blood cell count (Ditzel et al., 2000). Gamma and alpha isoforms show no homology, but they have a common function where both isoforms carry out the enzymatic delivery of aminoacyl tRNAs to the ribosome.

3. Splicing factor, arginine/serine-rich 11 (SFRS11):

SFRS11 was selected once from a control disease patient in NPH group by PD, once in SZ and once in bipolar disease group by protein array. Blast nucleotide alignment of two sequences yielded a 100% match where the whole sequence from PD was found within the sequence obtained from protein array. Protein array mapped to clone NM_004768.2, within bases 1069 to 1952, whereas the clone from PD mapped between bases 1454 to 1746.

4. Myosin light polypeptide 6B and myosin IXB (MYO9B)

Myosin is a hexameric ATPase cellular motor protein. It is composed of two heavy chains, two nonphosphorylatable alkali light chains, and two phosphorylatable regulatory light chains. MYL6B was identified in 1 BPD and 1 control disease patient (EPI group) by protein array. It encodes a myosin alkali light chain expressed in both slow-twitch skeletal muscle and in non-muscle tissue.

MYO9B (myosin IXB) was identified from a BPD patient by the PD method. MYO9B is also a motor protein with an unconventional role in actin remodeling. It interacts with calmodulin (Post et al., 1998) and moves on actin filaments (Inoue et al., 2002). Like other CALM1-containing myosins, it exhibits maximal velocity of actin filaments in the absence of calcium. There is recent evidence of MYO9B involvement in celiac disease, systemic lupus erythematosus, and rheumatoid arthritis and MYO9B is recognized as a general risk factor for autoimmunity (Sánchez et al., 2007).

5. Ribosomal proteins L17, L24 and S12, S27a

Ribosomes consist of a small 40S subunit, and a large 60S subunit, that are composed of 4 RNA species and approximately 80 different proteins. RPL17 was identified by PD in 2 SZ patients exclusively. It encodes a ribosomal protein that is a component of the 60S subunit. RPL24 was selected from 1 MS patient. Similarly, RPS27 was detected by protein array from 2 SZ patients. RPS27 encodes a component of the 40S subunit of the ribosome. RPS27 protein belongs to the S27E family of ribosomal proteins. It is located both in the cytoplasm as a ribosomal component, and also in the nucleus. RPS12 is also a component of the 40S subunit and it was detected in 2 SZ and 1 disease control (EPI group).

6. Zinc finger proteins

Zinc finger proteins (ZNF) belong to transcriptional factors and have diverse functions including DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding (Laity, Lee and Wright, 2001). ZNF410 and ZNF85 were selected by PD assay from 1 SZ and 1 MS patient, respectively. ZNF259 was selected from 2 BPD patients and ZNF358 from 1 SZ and 1 disease control (EPI group) by the protein array.

7. RNA binding proteins NOB1 and RD

NOB1 was selected from 1 MS patient by the PD assay, while RD (also-called negative elongation factor E) was identified exclusively in 3 BPD patients by the protein array. Both are RNA binding proteins although RD has not been shown to bind RNA. Interestingly, it is encoded by a gene that is located to the major histocompatibility complex (MHC) class III region.

6.5 Summary

1. RZPD protein array yielded 20 disease-specific clones. When all clones with significant intensities (including non-disease-specific ones) were compared to PD assay results, it was found that several of them encode proteins that belong to the same families, including highly homologous pairs.

2. The most significant sequence homology (100% match according to Blast alignment) was observed in the arginine/serine-rich 11 splicing factor. It was selected from NPH disease control group by PD assay. However, it was identified in 1 SZ and 1 bipolar patient, and was classified as a disease-specific hit according to protein array.

3. Eukaryotic translation initiation factor 4A (isoforms 1 and 2) was identified by both methods as a SZ-specific autoantigen candidate clone.

CHAPTER 7

DISCUSSION

7.1 GENERAL DISCUSSION

Several etiological theories re being discussed for psychiatric disorders like SZ and BPD. None of the prevailing views, however, can fully explain the different appearance and prognosis in individual patients. Technological advances and accumulating evidence that show immune dysfunction and the presence of autoantibodies in patient sera and CSF have highlighted the autoimmune hypothesis on SZ and bipolar disease.

The aim of this study was to investigate the autoimmune reactivity within CSF, based on preliminary western blot data from our lab showing the immune response against unknown targets in the CSF of depression patients (unpublished observation). Based on the evidence of possible autoimmune etiology, the research has expanded to include major psychoses like of SZ and BPD. The strategy applied is called immunoproteomics, where disease-specific autoantibodies against brain proteins were selected using PD.

This is a pioneering work based on cDNA PD selection of CSF, in order to test the autoimmune hypothesis for affective disorders and SZ. The complete approach was extensively tested and optimized to overcome limitations such as low immunoglobulin content, a limited volume of CSF and a high false positive ratio of PD selection. During selection and validation, we studied all samples individually

rather than pooling them, as the risk of diluting subtle autoimmune response within the heterogeneity of the population was too strong.

The protocol optimized for this work is sensitive enough to detect possible autoantigenic targets at nanomolar levels. However, there were other limitations of the technique that were unavoidable. The foremost drawback was the accumulation of the false positives along with the true hits. The experimental configurations often offered limited possibilities of finding any positive control that could directly single out the background or sticky clones right after the selection, before sequencing. There was no way to sort out which clones were real candidates by just looking at their properties or amino acid sequences. Running a phage ELISA for all the hits to see if they were real was not practical due to the limited CSF amount. For this reason, several control parameters were applied that compared enrichment values to the background between selections. It is plausible to expect that any given clone with a high affinity to the target should be able to dominate the final eluate. However, it was often observed that several true hits were represented in small percentages within the eluate, and with a low frequency among the whole disease group. This may suggest that such high affinity clones do not necessarily acquire efficient bacterial expression and, furthermore, all clones were not represented equally among the original library reflecting their abundance in that tissue.

The confidence in discriminating true hits from others was based on the fact that the probability of obtaining the same clone, even once at different target selections is extremely low. A complementary argument was to select the same clone multiple times, thus having high hit frequency mainly within the targeted disease group.

However, successful antigen identification is based on many factors like the type of the epitope, existence of the sequence as a known cDNA product and the reaction stoichiometry between target phage and the autoantibody. It is therefore, advised to use PD for antigen selection in conjunction with other methods (Owens et al., 2006). Furthermore, PD selection basically mimics natural interactions as the partners are in a non-reduced environment, creating a tendency to detect nonlinear (conformational) epitopes that are targets of the B-cell mediated immune response. Selection via protein arrays creates the possibility of screening exposed linear epitopes, which resemble antigens processed as peptides and become targets of T-cell mediated immune recognition. Accordingly, a protein array was applied in this study consisting of up to 27,648 proteins derived from expression verified, full-length as well as shorter cDNA clones. The protein spots were printed on PVDF membranes in duplicate so that they reached a total of 55,296 spots.

PD and protein array yielded different sets of candidate clones. It was observed that, PD selection was more efficient in identifying transmembrane proteins, while protein array mostly yielded soluble proteins. Interestingly, there were clones common to both methods but the hits were not frequent enough to qualify as autoantigen candidates, or they were listed as background clones in the PD method but appeared as hits in the protein array. Such diversities in the results can partially be explained by the principal differences in the methods. In addition, a human sample is a heterogeneous material, thus, either technique may provide a better outcome compared to the other. One common aspect was that, in addition to the known cDNA products, both methods yielded a large number of unknown gene products, including several 3'UTR sequences.

For verification of the significance of the selected candidate cDNA products in raising an autoimmune response, phage ELISA was applied. In this method, CsCl purified phage particles serve as an antigenic target for CSF autoantibodies. When successful, this method is very practical as it bypasses cloning and allows direct application of purified phage as a target. We obtained differences between positive CSF and positive lysate, compared to control lysates, despite a constant substantial background noise.

As an alternative method of validation, a conventional ELISA was employed using recombinant peptide instead of purified phage. The most significant candidate was cloned in an expression vector. For this purpose, the sequence of the phage genome obtained from PCR amplified insert was used, which covered 61 amino acids from the N-terminal of the candidate autoantigen target. In addition, we compared the specificity of this reaction by using peptides mapping to the same N-terminal sequence from the other family members of the candidate protein. Additionally, we tested positive and negative CSF samples for their response to recombinant peptide, which showed a trend of higher reactivity among positive CSF than the negative group, even though overall signal intensities were weak ($p<0.15$, two-tailed test).

Immune responses from control peptides were neutral while the response to the selected epitope was significantly strong ($p<0.05$, two-tailed test). These results suggest a specific immune response against the epitope mimicking the discoidin domain of the candidate autoantigen, and the sequence obtained by PD was significantly more antigenic than its homologues.

A complementary goal was to explore whether the obtained autoantigens could serve as biomarkers for affective disorders and SZ. Here the sample quality is the key factor, having a major impact on the success of biomarker research (Holland, Pfleger, Berger, Ho and Bastaki, 2005). Apparently, the population is heterogeneous, dynamic and affected by infinitely diverse environmental influences. Therefore, finding a sample for a disease group covering every possible phenotype is not easy (Petricoin, Belluco, Araujo and Liotta, 2006). Similarly, to set a clear-cut definition for "normal", especially in the field of psychiatry, is rather subjective. In this study, it was important that patient and control demographics met the aim of selection. In addition, the epidemiological history and medical records were thoroughly documented and analyzed by our clinician partners to exclude the secondary effects of antipsychotic drugs. Finally, there wasn't any direct correlation between patient history or patient data, and our screening results.

Studying disorders with unclear psychopathologies and poor diagnostic efficiency leads to limited, if not conflicting results that are often found in the literature. Most of the findings refer to a subgroup of patients who are distinguished from others with their predisposition to autoimmune disorders, genetic background or other proposed etiologies. In other words, the success in unraveling a disease etiology is dependent on good sampling, and yet the observations usually reflect only the group of patients studied. Accordingly, evidence on the disease etiology for SZ or bipolar patients cannot be reflected universally for all patients, unless the diagnostic criteria for the analyzed sample group were efficient and objective.

7.2 Candidate autoantigens selected from AFD and SZ patients

7.2.1 CASPR (Contactin Associated Protein, or Cell Recognition Protein)

Autoantibodies specific for contactin associated protein 4 (Caspr4) were in the CSF of 4 patients (3 SZ and 1 BPD), out of 11 (6 SZ and 5 BPD). Inflammatory/autoimmunity related control disease samples from MS yielded 1 Caspr4 positive patient, whereas non-inflammatory control group yielded a positive result from a headache patient.

Nucleotide sequences from Caspr4 inserts were mostly overlapping, only having different sizes. The longest insert encoded 143 amino acids (aa30-aa173) at the N-terminus of Caspr4. The Blast search identified the sequenced clone as both Caspr4 and Caspr3 as they have a high sequence homology (85%). Therefore, Caspr4 and Caspr3 were both considered as candidate autoantigens.

Caspr is the first and essential molecular constituent in the generation of paranodal junctions, identified from rats as contactin-associated protein (Einheber et al., 1997). As depicted in Figure 7.1, Caspr is a major transmembrane factor required for the integrity of septate/like junctions of the paranode.

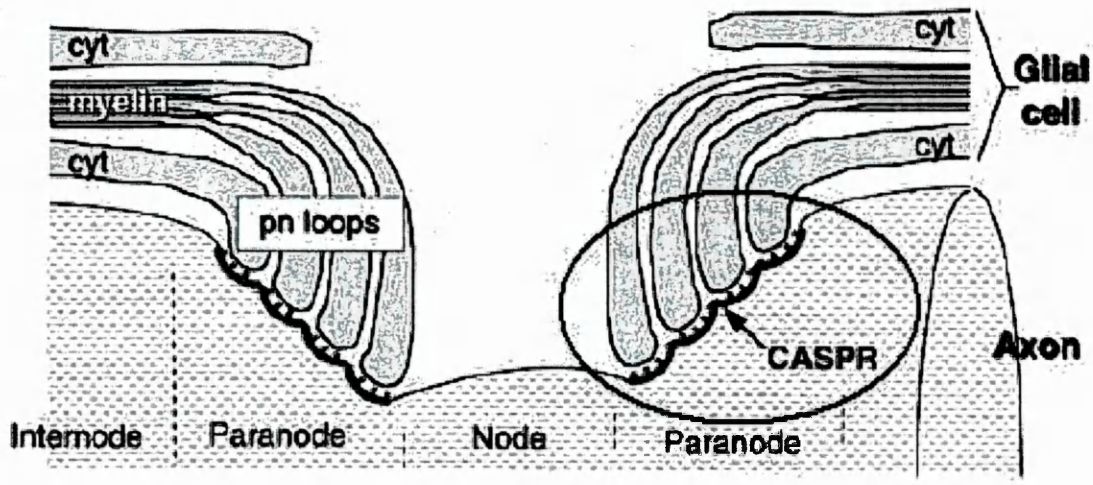


Figure 7.1 Longitudinal organization of the myelinated axon. Three distinct domains, internode, paranode and node of Ranvier are depicted with a focus on Caspr as a major transmembrane component of the septate-like junctions that protrude from axons towards the glial membrane at the paranodes (schematic structure showing the nodal region and location of Caspr is adopted from Einheber et al., 1997).

The internode, the paranodal region, and the node of Ranvier are three domains that form between axons and myelinating glial cells, i.e., Schwann cells in the peripheral nervous system and oligodendrocytes in the central nervous system (Peters et al., 1991; Salzer, 1997). In the vertebrate nervous system, myelination is required for efficient propagation of action potentials through saltatory conduction that depends on the distribution of voltage-gated channels among those three domains. The myelin sheath is attached to the axon at both sides of the nodes of Ranvier, forming a septate-like junction known as the axoglial or the paranodal junction (Rosenbluth, 1995).

Contactin Associated proteins belong to the NCP (NeurexinIV, Caspr, Paranodin) family (Bellen, Lu, Beckstead and Bhat, 1998) as a distinct subgroup of neurexins that are cell adhesion molecules at the synaptic cleft (Craig and Kang, 2007). In myelinated axons, Caspr is co-localized with the neural cell adhesion molecule

contactin at the paranodal junctions between axons and the terminal loops of oligodendrocytes in the CNS and myelinating Schwann cells in the PNS (Einheber et al., 1997; Menegoz et al., 1997; Rios et al., 2000). Contactin is a glycosylphosphatidyl inositol (GPI)-anchored member of the immunoglobulin gene superfamily (Ranscht et al., 1988; Gennarini et al., 1989), which promotes neurite outgrowth and fasciculation, and possibly synapse formation and maintenance (Faivre-Sarrailh and Rougon, 1997; Berglund et al., 1999). Binding of Contactin to an isoform of neurofascin (NF155) on glial loops (Charles et al., 2002) is inhibited by Caspr (Golan et al., 2003). It is expressed by neurons and oligodendrocytes but not Schwann cells (Einheber et al., 1997). The extracellular domain of Caspr binds to contactin in a cis interaction to form an adhesion complex (Peles et al., 1997). Caspr cannot be exported from ER to plasma membrane without this interaction (Faivre-Sarrailh et al., 2000), as shown by experiments on contactin null mice where Caspr was confined within neuronal cell bodies (Boyle et al., 2001). It is suggested that the interaction between Caspr and Contactin is necessary to anchor the whole complex on the axonal cytoskeleton, as Contactin is lost when Caspr is also lacking (Gollan et al., 2002). Furthermore, when both Caspr and Contactin are absent, the space between axon and paranodal loops gets larger (Boyle et al., 2001).

On the other hand, the cis interaction that takes place between Caspr and Contactin when they are in the same membrane does not necessitate their association during the myelination process. They are both diffusely expressed until later stages of myelination. On myelinated fibers Caspr accumulates at the paranodes, starting from the most mature myelin sheets, whereas Contactin is almost undetectable (Einheber et al., 1997).

The Caspr-Contactin complex is found in the paranodes of the optic nerve, although free contactin is located adjacent to the nodes of Ranvier (Rios et al., 2000).

Contactin is found in two glycoforms: high molecular weight (HMW) and low molecular weight (LMW), depending on the level of glycosylation. Caspr binds to LMW Contactin and acts as a chaperone that brings it to plasma membrane, thereby reducing the number of HMW isoform. HMW Contactin can be found in extracellular membrane without Caspr. Thus, Caspr was shown to have a major role in determining the localization of LMW Contactin glycoform (Gollan et al., 2003). Moreover, Caspr can only be poorly extracted from neurons by non-ionic detergents indicating that it is a constitutive component of the axon cytoskeleton (Einheber et al., 1997) or its association with Contactin guides the complex to detergent insoluble domains (Faivre-Sarrailh et al., 2000).

Initially Na^+ channels cluster adjacent to the locations where the processes extend from oligodendrocytes (Boiko et al., 2001) and myelinating Schwann cells (Wabnick et al., 1996). Caspr accumulates at paranodes, where each ring that Caspr associates with, represents one turn of the myelin wrap (Rios et al., 2000). In correlation to this function, Caspr was shown to have an abnormal distribution in multiple sclerosis (MS) (Wolswijk and Balesar, 2003).

In Caspr null mice, paranodal junctions cannot form properly and Contactin is absent at this site (Bhat et al., 2001). Additionally, Gollan et al., 2003, showed that, in the absence of caspr, HMW Contactin dominates and localizes at the nodes instead of paranodes in the CNS. This is in agreement with Bhat et al., 2001.

Caspr3 and Caspr4 were identified as two novel and closely related members of Neurexin IV/Caspr/paranodin (NCP) family proteins (Spiegel et al., 2002). There is only one study on Caspr3 and Caspr4, namely the original paper from Spiegel et al. that reports the structure, *in-situ* hybridization and immuno-localization of Caspr3 and Caspr4. Surprisingly, this study shows that Caspr2, Caspr3 and Caspr4 do not bind to Contactin.

Similar to Caspr and Caspr2, both Caspr3 and Caspr4 share a structural resemblance to neurexins, as they have a large extracellular domain, single transmembrane domain and a short cytoplasmic domain. Unlike Caspr, but similar to Caspr2 and other neurexins, both Caspr3 and Caspr4 have a binding site for PDZ domains at their carboxy termini. Remarkably, caspr3 and caspr4 have a 70% complete amino acid sequence homology, which is the highest among all caspr proteins. The domains of all members of the Caspr family are given in Figure 7.2.

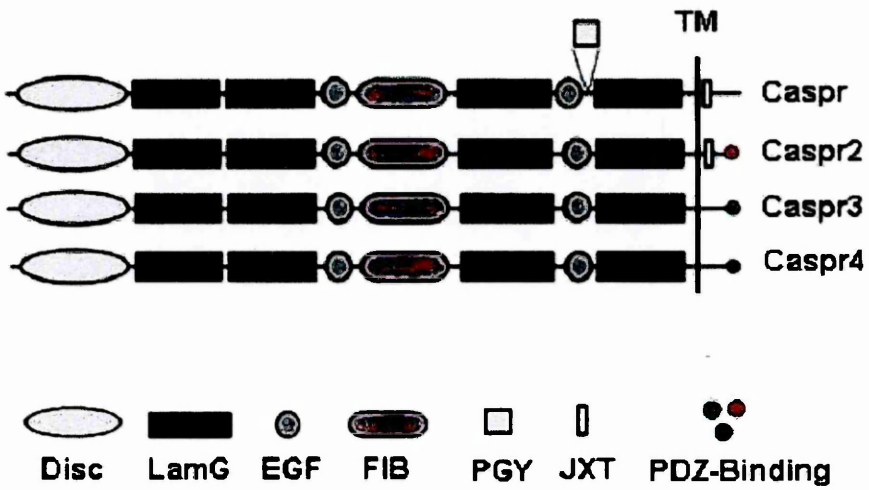


Figure 7.2 Structure of Caspr family proteins. All Caspr proteins contain a discoidin-like domain (DISC), four laminin G (Lam G) domains, a region similar to fibrinogen (FIB), two EGF repeats and tyrosine residues. Proline glycine repeats (PGY) are found only in Caspr. Caspr3 and Caspr4 have a short cytoplasmic domain that contains a carboxy-terminal PDZ binding domain. Scheme modified from Spiegel et al., 2002.

According to Spiegel et al., both Caspr3 and Caspr4 are predominantly expressed in the human brain and the spinal cord. The highest Caspr4 expression was shown to be in subthalamic nuclei, substantia nigra, corpus callosum and medulla, whereas Caspr3 was found highly expressed in temporal and frontal lobes, corpus callosum, medulla and hypothalamus. High expression of Caspr 3 was reported in oligodendrocytes in the corpus callosum and within the cortical white matter, but it was not detectable in Schwann cells. Reciprocally, expression of caspr4 was undetectable in oligodendrocytes. *In situ* hybridization results implicated that Caspr4 localizes in the cell bodies and the dendrites in the brain, whereas Caspr3 was reported to be located along axons and in oligodendrocytes.

Both soluble and transmembrane forms of Caspr3 have been reported to have a role in glia-neuron interactions. In fact, Caspr3 was suggested as a candidate regulatory factor clustering Na^+ channels during the early phase of CNS myelination (Spiegel et al., 2002), which is a similar function to axotactin production by glial cells in Drosophila (Yuan and Ganetzky, 1999). Furthermore, Schwann cells regulate Na^+ channels in the PNS by direct contact, not via a soluble factor, which may explain the undetectable levels of Caspr3 in Schwann cells.

Interestingly, all Caspr proteins are distinguished from other neurexins by two distinct domains in the extracellular region, namely the discoidin domain (also-called blood coagulation factor FA5/8C) and the fibrinogen/like domain (Poliak et al., 1999, Peles et al., 1997).

Our results indicate that the candidate autoantigenic peptide maps to the discoidin domain of Caspr4/Caspr3. With respect to this, we focused on the properties and proteins that possess discoidin domain (DISC domain).

The discoidin domain is identified by an adhesion protein called discoidin that is extracted from the Dictyostelium discoideum (slime mold) cell. Identification of two C-terminal repeats of blood coagulation factors V and VIII (Jenny et al., 1987; Wood et al., 1987 respectively) being similar to N-terminal region of discoidin, helped define a new extracellular domain called DISC or FA5/8C. However, the N-terminal 40 amino acids are much less conserved in DISC domain than FA5/8C. Proteins with a discoidin motif include: neurophilins, which are receptors of semaphorins involved in axonal growth, neurexins, which mediate cell-to-cell contacts and blood coagulation factors V, VIII and XLRS-1 (Franco-Pons et al., 2006). The proteins that have the discoidin domain are given in Table 7.1.

Table 7.1 Proteins that contain the DISC domain (adopted from Baumgartner et al., 1998)

Name	Accession	Synonym	Function
Discoidin II	P42530		
Factor V, VIII	P12259, P00451		Blood coagulation, phospholipid binding
Neurophilin	AF018956	A5 Antigen	Ca^{2+}- dependent cell adhesion, cell recognition in the nervous system, receptors of semaphorins involved in axon growth
Discoidin domain receptor, Receptor Tyrosine kinase	Q08345	DDR1, TrkE, RTK6, CAK	Cell-cell interaction and recognition
Neurexin IV	U87223	CASPR	Cell adhesion, cell junction

Our findings suggest that IgG from the CSF of some SZ and BPD patients has affinity towards 60 amino acids, located at the N-terminus that map within the discoidin domain of Caspr4/Caspr3. Three out of 6 SZ patients and only 1 BPD patient that were selected by PD produced positive response by ELISA, suggesting a more pronounced titer within the SZ disease group.

Remarkably, a very recent study reported that the discoidin domain receptor1 (DDR1) gene is a susceptibility locus for SZ (Roig et al., 2007). In correlation with several lines of evidence suggesting that the myelin alterations predispose individuals to SZ (discussed in section 8.3), Roig et al., have identified DDR1 as a myelin gene in the human oligodendroglial cell line that is located within one of the best-supported chromosomal regions for susceptibility to SZ, namely 6p24–21 that contains the major histocompatibility complex I (MHC I) (Owen et al., 2004). Table 7.2 lists the putative susceptibility genes and their loci within this region.

Table 7.2 Putative susceptibility genes for schizophrenia within locus 6p24-21.

Gene	Loci	Reference
Dysbindin 1 (DTNBP1)	6p22.3	Kendler et al., 2004
Myelin oligodendrocyte glycoprotein (MOG)	6p22.1	Liu et al., 2005
Tumor necrosis factor alpha (TNF-α)	6p21.3	Saviouk et al., 2005, Hashimoto et al., 2004, Schwab et al., 2003
Notch type-4 receptor (NOTCH4)	6p21.3	Glatt et al., 2005; Prasad et al., 2004; Luo et al., 2004; Skol et al., 2003; Wei et al., 2000
Tenascin B (TNXB)	6p21.3	Wei et al., 2004
Discoidin domain receptor1 (DDR1)	6p21.3	Roig et al, 2007

DDR is a novel subfamily of receptor tyrosine kinases (RTK), distinguishable from others due to its homology domain to discoidin. DDR family proteins possess a 160 amino acid long DISC homology domain, a single transmembrane region followed by an extended juxtamembrane region and a catalytic tyrosine kinase domain. DDR1 can be activated by all types of collagens which are main components of the extracellular matrix (EM) (Vogel et al., 1997; Shrivastva et al., 1998). DDR1 activation is similar to other RTKs in that, native collagen induces DDR1 dimerization and transphosphorylation. DDR1 expression is most abundant in the brain, but it can also be found in keratinocytes, the kidney, lung, breast, muscle and thyroid follicles (Laval et al., 1994; Di Marco et al., 1993; Perez et al., 1994; Zerlin et al., 1993).

DDR1 null mice are viable but smaller in size. *In vitro* studies revealed that DDR1 is involved in cell proliferation and adhesion (Curat and Vogel, 2002), cell migration (Hou et al., 2001, Kamohara et al., 2001) and disintegration of EM by matrix metalloproteinase (MMP) activity (Hou et al., 2001). In particular, it is notable that the interaction of DDR1 with collagen of the extracellular matrix allows leukocytes to migrate through tissues during the intracellular signaling process (Kamohara et al. 2001).

Notably, DDR1 is expressed by a subpopulation of glial cells in the white matter in the adult murine brain (Zerlin et al., 1993). DDR1 mRNA localizes close to myelinated fibers. Additionally, the cellular pattern of DDR1 positive cells, which resemble oligodendrocytes, is prominent during myelination. On the other hand, myelination markers like myelin associated glycoprotein (MAG), or myelin basic protein (MBP) have not been shown to co-localize with DDR1 (Franco-Pons et al., 2006).

7.2.2 Myelination related factors that may be involved in the pathology of SZ as candidate autoimmune targets in the CNS

In the light of our findings, that suggest an autoimmune activity towards myelination-related factors, we performed a brief functional study, in which the genomic expression profile of selected candidate Caspr4 and three others, Caspr1, MBP and DDR1 (SZ susceptibility loci bearing myelin gene with discoidin domain), were analyzed. For this purpose, the abundance of mRNA in brain and spinal cord of mice, from PND1 to PND42, were evaluated by quantitative PCR, as the myelin maturation takes place actively within this period.

We observed a gender difference in the pattern of expression of Caspr4 and DDR1, where female mice were found to express significantly higher levels of

mRNA in the brain compared to the males during the first week of life ($p<0.05$, t-test). For both sexes, Caspr4 and DDR1 were downregulated afterwards whereas MBP and Caspr1 were upregulated, reaching peak transcript concentrations on PND14 and PND28 respectively. In spinal cord, the only difference in expression pattern was at PND7, when Caspr4 was upregulated in female mice in contrast to males. A striking difference between brain and spinal cord became evident when samples from both sexes were pooled and analyzed together. Caspr1 and DDR1 expression pattern in spinal cord was significantly different from the expression of MBP and Caspr4, which resembled the expression difference in the brain of females and males. Specifically, both Caspr4 and MBP expression were high between PND1-PND7, and then started to get downregulated until stabilization at PND14-PND21. The opposite was observed for Caspr1 and DDR1, where both transcripts reached maximal concentration at PND14 and later stabilized after a mild drop. During PND1-PND42 Caspr1 mRNA was found to be significantly abundant, reaching up to 1.2% of the number of housekeeping genes at PND28, whereas, in spinal cord, both Caspr1 and Caspr4 mRNA were more abundant then MBP and DDR1. This study provided new information on similar or contrasting patterns of expression that may suggest a possible interplay or partnership in the relevant neuronal maintenance mechanism.

7.2.3 Support for the disconnectivity hypothesis for SZ and AFD

Identification of members of Caspr family proteins as candidates for autoimmune responses emphasizes a new aspect of the disconnectivity hypothesis that is based on downregulation of junction and nodal proteins like the peripheral myelin protein (PMP22), proteolipid protein (PLP), myelin associated glycoprotein (MAG) in SZ. A substantial downregulation of myelin-associated genes, as demonstrated in microarray studies, may be accompanied by a secondary impairment during white matter expansion that would distort axo-glial organization and interfere with myelination. As a result, abnormal oligodendrocytes among myelinated fibers may become the target of immune response and be engulfed by astrocytes, leading to loss of oligodendroglial cells. In fact, a reduced oligodendroglial density up to 22% (Hof et al., 2002) was shown in *post mortem* prefrontal cortex (PFC) in SZ, bipolar affective disorder, and major depression (Rajkowska-Markov et al., 1999; Orlovskaya et al., 2000; Torrey et al., 2000; Cotter et al., 2000). Such studies, showing a loss in glial cell density and quantitative reduction in white matter, confirm a major disintegrity in oligodendrocytes and myelin-related proteins in the SZ.

Our results are therefore consistent with and supportive of the suggested correlation between the disconnectivity hypothesis of SZ and the stability of the myelination by myelin protein abundance, proper functioning of oligodendrocytes and adhesion-junction proteins maintaining nodes of Ranvier, paranodes, juxtaparanodes and internodal regions.

7.2.4 Other autoantigen candidates selected by phage display

7.2.4.1 Growth Arrest Specific Protein 7 (GAS7)

We identified Gas7 as a candidate autoantigen from 2 SZ patients and 1 BPD patient. Additionally, 3 MS patients, one of whom was also reactive to Caspr4, were positive for Gas7.

Schneider et al. first identified growth arrest specific genes by the induction of Gas genes after serum starvation and density-dependent inhibition of growth (Schneider et al., 1988). The expression of growth arrest-specific (Gas and Gadd) genes is associated with the G0'-arrest cycle (Hass, 1994). Expression of growth arrest-specific (Gas) genes is observed during growth arrest in terminally differentiating cells during the development of peripheral nerves. Overexpression of GAS7 in undifferentiated neuroblastoma cell cultures promotes neurite-like outgrowth. On the other hand, inhibition of GAS7 in terminally differentiating cultures of embryonic murine cerebellum impedes neurite outgrowth from maturing Purkinje cells (Ju et al., 1998). The role of Gas7 in neuronal differentiation is characterized by adenovirus-mediated overexpression in PC12 cells, and quantification of the expression of various neuronal markers in the absence and presence of different concentrations of nerve growth factor (NGF) (Chang et al., 2005).

Gas7 interacts with F-actin and co-localizes with the terminal part of the actin microfilament in cells going through membrane outgrowth (Chao et al., 2005). Human GAS7 is located on chromosome 17p11.3, encoding a 48-kD protein structural domain which resembles transcription factor POU that is implicated in neuronal development, and synapsin1 (SYN1) which is involved in the modulation of the neurotransmitter release.

Interestingly, a recent study on Gas6, another member of the GAS family of genes, reports its role in increasing the survival rate of Schwann cells and neurons *in vitro* and its participation in autoimmunity in animal models. Furthermore, Gas6 protein concentration is elevated in the CSF of patients with chronic inflammatory demyelinating polyneuropathy (CIDP), suggesting its involvement in myelin repair (Sainaghi et al., 2008).

7.2.4.2 Talin

Talin was selected exclusively from 2 SZ patients out of a total of 6, and not from any of the control or MS disease samples (n=23). Talin is a large dimeric protein acting as an adaptor molecule that couples the actin cytoskeleton to integrins. Integrins are heterodimeric proteins with single pass membrane domains, and they belong to cell adhesion molecules (CAMs) that include immunoglobulins, cadherin, neurexins and neuroligand protein families as well (Benson, Schnapp, Shapiro and Huntley, 2000). As mentioned earlier, neurexins include the Caspr protein family. Talins are reported to be present at the neuronal synapse where they interact with, and activate phosphatidylinositol-(4)-phosphate 5-kinase type I (PIPKIγ) (Di Paolo et al., 2002, Ling et al., 2002). When talin–PIPKIγ interaction at synapses is disturbed, synaptic vesicle recycling and actin dynamics are impaired (Morgan et al., 2004). Integrin and PIPKIγ compete for talin (Barsukov et al., 2003); thus regulatory mechanisms that control binding of PIPKIγ to talin may be affected if talin is a target of autoimmunity.

7.2.4.3 Myelin basic protein (MBP)

Myelin basic protein (MBP) was found in 1 SZ, 1 BPD and 1 MS patient. We included MBP in the expression pattern analysis, together with other myelination-related and candidate autoantigen genes.

Myelin sheaths and the clustering of ion channels at the nodes of Ranvier facilitate efficient and fast action potential propagation in vertebrates. MBP is the major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the nervous system. It is, however, not only a structural protein that works as an insulator around axons, but it also actively regulates several neuronal processes (Schafer et al., 2004) including ion channel expression and localization (Poliak and Peles, 2003; Salzer, 2003).

MBP destruction throughout the nervous system is the main pathology of multiple sclerosis (MS) although the etiology remains largely unknown. It is well known that MS patients suffer from demyelination due to autoimmunity that eliminates myelin proteins faster than the re-myelination, yet autoantibodies against MBP cannot be efficiently isolated from CSF. Interestingly, only a subset of patients (in the range of 59%) show detectable Ig titers against MBP (Egg et al., 2001).

It is interesting that, according to PD selection, the SZ patient, who was responsive to MBP, has reacted with growth associated protein 43 (GAP43) as well. GAP43 is highly expressed in developing neurons (Perrone-Bizzozero et al., 1996) and remains at high levels in adult human brain at hippocampus and cortical regions thus hypothesized to be involved in synaptic plasticity and connectivity (Akers et al., 1985). Notably, in SZ brains GAP43 is found to be highly expressed in visual and frontal cortical regions (Perrone-Bizzozero et al., 1996). It can be

speculated that abnormal high expression of GAP43 might trigger autoimmune activity in CNS. A simultaneous autoimmune response against MBP might be a secondary effect or mutually independent.

7.3 Summary and Conclusion

Myelination and oligodendroglial function affects neural connectivity that facilitates the communication between multiple brain regions and circuits. White matter expansion in the prefrontal area is caused by increased myelination that is accompanied by gray matter reduction within a time frame of late adolescence and early adulthood. Abnormalities in myelination are negatively reflected on neural connectivity that would normally be compensated by the expansion of white matter during the reduction of gray matter. Several lines of evidence, most of which come from imaging studies, suggest that functionally disintegrated neural circuitry is the major abnormality in SZ as seen by a disconnection between prefrontal and posterior areas in the brain (Frith and Dolan, 1996).

Imaging techniques in the prefrontal cortex of SZ *post mortem* brain tissue showed myelin and oligodendroglia abnormalities (Miyakawa et al., 1972; Deicken et al., 1994). Additionally, structural impairments, shown on biopsy and autopsy samples from the frontal cortex of SZ patients, included reduced compactness and formation of the concentric lamellar of the myelin sheath (Orlovskaya et al., 2000, Torrey et al., 2000, Cotter et al., 2000). In general, impaired myelination can be attributed to developmental disturbances, oligodendrocytic dysfunction and cell death, or other regulatory deficits.

The first evidence about expression changes in myelin-associated genes in SZ was provided by microarray studies (Hakak et al., 2001). The results were reproduced by other studies where the genes related to myelination, and oligodendrocyte function was found to be downregulated (Dracheva et al., 2006; Sugai et al, 2005; Katsel et al., 2004; Stewart and Davis, 2004; Aston et al., 2004;

Tkachev et al., 2003). To sum up, the most prominent changes in protein expression in SZ were those that are involved in glial differentiation, myelin structure and adhesion proteins found in axoglial contacts (Katsel et al., 2005).

Our findings implicate that an epitope at the N-terminus of Caspr4 (also Caspr3, with 85% aa sequence homology), but not Caspr1 and Caspr2, is a possible target for an autoimmune response, at least in some cases of SZ.

Another notable implication is based on the importance of the identified epitope for autoantigen targeting, namely the DISC domain. Besides being reported as a SZ susceptibility gene and a myelin protein, DDR1 is a cell-surface receptor tyrosine kinase that gets activated by binding to fibrillar collagen through the DISC domain. Interestingly, it is the DISC domain that distinguishes Caspr family members from other neurexins. As a result, a further inquiry addressing the possibility of Caspr4/Caspr3 interacting with collagens is intriguing. Recent studies report that collagens have an important role in the formation of the node of Ranvier (Maertens et al., 2007; Occhi et al., 2005). It is suggested that in myelinating peripheral nerves, gliomedin (a transmembrane collagen family including collagen type XIII and XVII) binds to axonal adhesion molecules like neurofascin and the neuron-glia-CAM-related cell adhesion molecule (NrCAM) to mediate sodium channel clustering at the nodes of Ranvier.

It is therefore noteworthy to underline the possible implication of proteins carrying the discoidin domain with regard to SZ. In other words, instability in a subset of proteins with the discoidin domain might have an adverse effect on myelination, as well as possible interactions with collagen, thus, eventually causing a diverse neuropathology.

When SZ is compared to demyelinating disorders like multiple sclerosis and metachromatic leukodystrophy (MLD), many similarities become clear considering the psychiatric and cognitive discrepancies (Davis, 2003). In the case of MLD, a rare inborn disease, the accumulation of sulfatides in the CNS and PNS causes demyelination that begins in the periventricular white matter and corpus callosum. Interestingly, late adolescence and early adulthood onset type MLD displays mainly psychiatric symptoms including auditory hallucinations, emotional liability, loss of concentration, thought fragmentation and poor concentration, which begin after age 16 and may lead to a misdiagnosis of SZ. It is remarkable that psychosis symptoms are observed solely when the disease is centered in the frontal lobes, before it spreads to the rest of the forebrain and is replaced by disorders of movement, posture late dementia (Hyde et al., 1992).

Caspr4 and Caspr3 are novel proteins, which have yet to be studied. Nevertheless, based on the high expression of Caspr3 in oligodendrocytes and within the cortical white matter, it can be hypothesized that destabilization of caspr3, probably by a temporal autoimmune attack, might be detrimental for a group of susceptible people, especially during white matter expansion between adolescence until early adulthood. Such alterations might form the base for schizoaffective symptoms that surface later in life, albeit with a subtle clinical association with a chronic autoimmune pathology. The lack of information on changes in their expression patterns during onset restricts us from providing further insight into their possible impact on the pathogenesis of SZ.

Our results additionally show that cDNA PD can be used in the identification of autoantigens in inflammatory and autoimmune diseases of the CNS. This is especially the case when the causal mechanism of the CNS specific IgG response and the nature of the target are unknown. cDNA library based-selection was the most applicable system, recapitulating the heterogeneity of the Ig content in the CSF and selecting peptides and proteins that exist in nature. Every round of selection generates a subpopulation of high affinity binders from the initial recombinant pool. The PD technology, indeed, introduced great flexibility to this project in adjusting stringency and other selective criteria. The manual application of selection by panning is somewhat time-consuming and laborious. The whole system could, however be scaled up as a high-throughput system if there were an opportunity for automation.

Consequently, our approach has proven effective for finding autoantigens as candidate biomarkers. We cannot, however, claim that Caspr4 or Caspr3 are absolute biomarkers for SZ and BPD. Furthermore, we agree that it is more reasonable to assume that an "array" of biomarkers spanning the profoundly heterogeneous population, will give a better assurance than one single biomarker (Gulmann, Sheehan, Kay, Liotta and Petricoin, 2006). Actual confirmation of their potential to act as biomarkers depends on understanding the molecular pathology showing what happens to latent patients under an acute attack of elevated caspr4 and caspr3 autoantibodies. It also depends on whether this can feasibly be detected when the acute phase is over and the major symptoms start to appear.

This study extended our knowledge of the role of myelination with regard to neuropsychiatry and supports the disconnectivity hypothesis of SZ. It is intriguing to consider that autoimmune targeting towards Caspr3 and Caspr4 proteins could

directly or indirectly impair myelination and, thereby, trigger schizoaffective symptoms in some cases of SZ and BPD. Further efforts, however, have to be made to gain more insight from our findings. These may include developing mouse models that are subjected to high titers of Caspr3 and Caspr4 antibodies during white matter maturation to observe changes in neuro-psychiatric conditions.

Eventually, we are suggesting a concept that connects autoimmunity to myelin and oligodendrocyte dysfunction with respect to the neuroetiology of SZ and AFD. Future studies focusing on revealing other factors that render autoimmunity towards destabilization of myelination, would provide important new insights into the current understanding of affective disorders.

APPENDICES

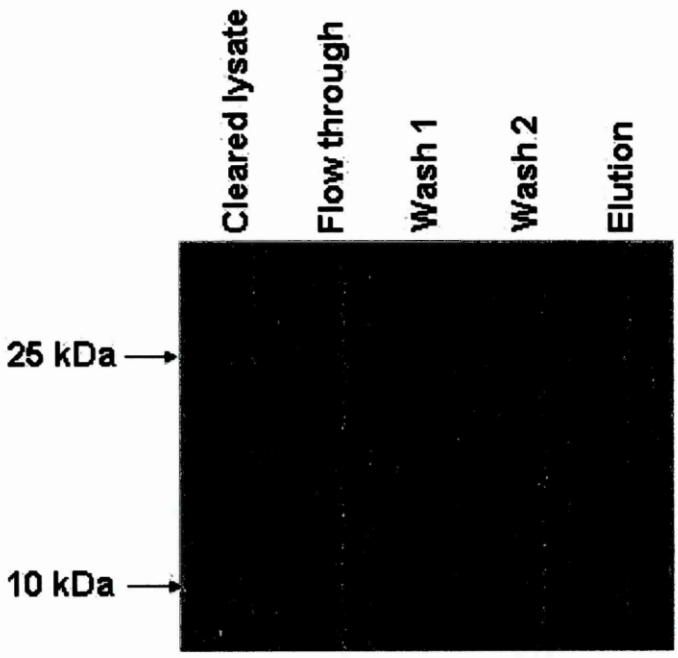
A1. Proposed etiologies and literature references

SZ Etiology	References
Neurodegeneration	Lieberman et al., 1999
Neurotransmitter system	Garbutt et al., 1983, Seeman et al., 1987, Dean et al., 2000;1999, Ohnuma et al., 1999, Tallerico et al., 2001, Tandon 1999, Taylor et al., 1998
Viral infection	Jones et al., 1998; Yolken et al., 1995, 2001, Leweke et al, 2004, Pelonero et al., 1990, Dickerson et al., 2003
Neurodevelopmental	Raine, 2006, Hornig et al., 2002, Toro and Deakin, 2007
Genetic predisposition	Mowry et al., 2001, Mueser et al., 2004, Tsuang et al., 2000
Autoimmunity / immune dysfunction	Noy et al.,1994; Fontana et al., 1980; Knight et al., 1992; Müller et al., 2000, Nunes, 2005, Lin et al., 1998, Altamura et al., 2003, Amital. et al., 1993; Jones, Mowry, Pender and Greer, 2005, Ganguli, Brar, Chengappa, Yang, Nimgaonkar and Rabin 1993; Henneberg et al., 1993, 1994, Legros et al., 1985, Ganguli, Brar and Rabin 1994; Wright et al., 1993; Logan et al., 1970; Kirch et al., 1993, DeLisi et al., 1986; Sirota et al., 1990; Hornig et al., 1999, 1996, Schwarz et al.,1998, Abramsky et al., 1978

A2. Detailed patient data

Disorder - Patient	IgG µg/ml	Albumin mg/l	Total protein mg/l	Gender	Age	IgG OCB CSF/serum	Total cell count/µl	Leukocyte number/µl	Erythrocyte number/µl	Local IgG/IgA/IgM synthesis
SZ-076/97	24,6	265	587	M	50	+ / +	6	n.a	n.a	- / - / -
SZ-369/98	53	348	493	F	43	- / -	1	n.a	n.a	- / - / -
SZ-454/00	22,7	147	280	F	34	- / -	1	n.a	n.a	- / - / -
SZ-166/02	28,7	230	420	M	56	- / -	5	n.a	n.a	- / - / -
SZ-464/02	45,6	424	520	F	24	- / -	4	n.a	n.a	- / - / -
SZ-244/04	23,3	166	278	F	61	- / -	1	1	0	- / - / -
BPD-160/98	14,7	140	229	M	21	+ / -	3	n.a	n.a	- / - / -
BPD-071/02	25,4	231	320	F	52	- / -	2	n.a	n.a	- / - / -
BPD-365/04	20,3	188	298	F	48	- / -	2	1	1	- / - / -
BPD-131/05	18,9	137	199	F	64	- / -	1	1	0	- / - / -
BPD-203/05	11,9	161	223	F	46	- / -	3	1	2	- / - / -
MDD-312	43,3	303	485	n.a	n.a	- / -	3	n.a	n.a	- / - / -
MDD-Lo	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
MS-70004	84,2	403	687	M	41	+ / -	1	1	0	+ / - / -
MS-06705	52,3	306	601	F	40	+ / -	1	1	0	+ / - / -
MS-14905	44,7	213	396	F	43	+ / -	1	1	0	+ / - / -
MS-15005	108	275	554	F	40	+ / -	8	8	0	+ / - / -
MS-24605	54,9	294	507	F	26	+ / -	0	0	0	+ / - / -
MS-24705	151	443	781	F	31	+ / -	22	22	0	+ / - / -
MS-31205	59,4	281	591	F	45	+ / -	5	5	0	+ / - / -
MS-35305	51,2	228	538	F	39	+ / -	8	8	0	+ / - / -
EPI-014/00	26,6	225	304	F	25	- / -	1	n.a	n.a	- / - / -
EPI-477/01	34	205	340	M	17	- / -	3	n.a	n.a	- / - / -
EPI-326/02	62,3	371	640	M	70	+ / -	21	n.a	n.a	- / - / -
EPI-300/04	15,5	132	180	F	26	- / -	36	1	35	- / - / -
EPI-330/04	31,9	289	411	M	46	- / -	4	3	1	- / - / -
EPI-339/04	61	265	428	F	56	- / -	2	1	1	- / - / -
EPI-053/05	21,1	172	242	M	23	- / -	3	2	1	- / - / -
OND-01805	23	210	411	F	39	- / -	1	1	0	- / - / -
OND-19405	34,1	320	578	F	45	- / -	0	0	0	- / - / -
OND-25505	17,3	177	323	F	41	- / -	2	2	0	- / - / -
OND-41405	26,6	308	620	F	42	+ / -	0	0	0	- / - / -
NPH-2	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
NPH-3	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a

A3. Expression of Caspr4 peptide



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